Effect of custom-made probiotic chocolates on *Streptococcus mutans*, plaque pH, salivary pH and buffering capacity in children- A randomized controlled trial

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CERTIFICATE

This is to certify that the dissertation titled "Effect of custom-made probiotic chocolates on *Streptococcus mutans*, plaque pH, salivary pH and buffering capacity in children- A randomized controlled trial"is a bonafide work done by Dr.JANANI RG, Postgraduate student, during the course of the study for the degree of "Master of Dental Surgery" in Department of Pedodontics and Preventive Dentistry, KSR Institute of Dental Science and Research,Tiruchengode during the period of 2015-2018.

Date: **Dr. G.S. Kumar, M.D.S.,**

Place: Tiruchengode **Principal**

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TITLE OF DISSERTATION	Effect of custom-made probiotic chocolates on *Streptococcus mutans*, plaque pH, salivary pH and buffering capacity in children- A randomized controlled trial
PLACE OF STUDY	K.S.R Institute of Dental Science and Research
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NAME OF THE GUIDE	Dr. GeethaPriya PR, M.D.S
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Head of the Department **Signature of candidate**

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CONTENTS

INTRODUCTION

World market has tagged Indians to be chocophile, owing to the larger and faster growth in chocolate industries. In 2016, when chocolate sales were stagnant in other countries, India topped the chocolate market. Over 44% of chocolate consumers in India assume chocolates to be healthy on consumption. Owing to the increased incidence of chocolate sales, it's nearly impossible to completely eradicate it from consumers.[10]

Chocolate, a solid confectionery is a mixture of cocoa, fat and powdered sugar. Chocolates have been mainly classified based on their cocoa content into three categories- milk, dark, and white chocolate. Milk chocolate is solid chocolate made with milk in the form of milk powder, sugar, cocoa mass, cocoa butter and or vegetable fat. White chocolate is a confection based on sugar, milk, and cocoa butter without the cocoa solids. Dark chocolate is produced by adding fat and sugar to cocoa. Dark chocolate is a suspension of 65% to 70% cocoa particles and sugar in cocoa butter. Particle size of sugar and cocoa particles is controlled to <30 μm to avoid grittiness and obtain a smooth in-mouth flow. Milk chocolate differs from dark chocolate notably through the presence of milk solids (milk powder) and lower content of cocoa.[5]

Chocolates are sticky in nature and contain large amounts of fermentable sugar making them highly cariogenic.[19] Chocolate contains sucrose from sugar particles and lactose from milk making it a fermentable carbohydrate. However chocolate consumption in causing dental caries is debatable. Rugg-Gunn et al[75] found a positive association between chocolates and caries which were not statistically significant. It was concluded that chocolate consumption solely was not a strong factor for caries. Verakaki and Duggal[94] emphasized the varying cocoa content in chocolates was one of the major factors in determining the acidogenicity of plaque.

Dental plaque is a structurally and functionally organized biofilm. It has a diverse microbial composition that remains relatively stable over time. It can be beneficial depending on

the composition and behavior of their microbial residents. The formation of biofilm occurs by adherence of the primary colonizers on the tooth surface, followed by coaggregation of secondary colonizers. Streptococcus and Actinomyces species are the most abundant early colonizers of the soft and hard tissues of the oral cavity. The transition from oral health to disease is as a result of ecological shifts caused by changes in the host oral environment, which finally lead to microbial imbalance within these biofilms.[48]

Stephan (1940)[84] conducted an experiment with 10% sucrose rinse and recorded the pH at various time intervals. The pH Values plotted as graph showed that after the sucrose rinse, the pH drops rapidly reaching pH 5.5 (critical pH) within 2-5 minutes. The pH remained under the critical level for 10-30 minutes. Then it returned gradually to the resting pH level after 60 minutes. Unless there is additional ingestion of fermentable carbohydrates, the pH of plaque gradually returns to its resting pH of 6 to 7.[8] The change in pH after a sugar challenges is mainly due to rate of acid formation, movement of acids in and out of the plaque, rate of acid neutralization by the plaque, tooth substance, buffer systems and the rate of acid neutralization by the bacteria.[25] Frequent consumption of sugars leads to a selective increase in acidogenic and aciduric strains of the oral environment. Hence there is a shift in the demineralization/ remineralization balance toward mineral loss, leading to the caries lesion development.[24]

The new era of caries prevention aims at altering the oral environment and bringing back microbial homeostasis. The use of probiotics for preventing dental diseases is at an emerging front. Systematic review by Laleman et al[43] and Cagetti et al[12] have shown probiotics to be a potential agent that can reduce the *S. mutans* colony count.

The credits of using probiotics dates back to our ancestors, who considered curd as a functional food. It was in early 1900's when Dr Metchnikoff was fascinated on the health and

well being of Bulgarian population. It was attributed to their practice of yoghurt consumption. He named the *Lactobacillus* found in curd after the Bulgarians as *Lactobacillus bulgaricus.* Lilly and Stillwell in 1965 proposed the term probiotic. Hull et al in 1984 introduced the first probiotic bacteria - Lactobacillus acidophilus. Parker in 1974 defined them as: "organisms and substances which contribute to intestinal balance". FDA/WHO (2002) defined probiotics as 'Live micro-organisms which, when administered in adequate amounts, confers health benefit on the host'. Bacteria is accepted as probiotic if it has the ability to: (i) exert a beneficial effect on the host; (iii) withstand transport through the GI tract; (ii) withstand in a foodstuff maintaining viability (v) produce antimicrobial substances against pathogens; (iv) adhere to the intestinal epithelial lining and (vi) stabilize the intestinal microflora.[65]

. The preventive and therapeutic role of probiotics have been studied on different aspects of health including its effect on diarrhea,[89] lactose intolerance,[49] hepatic diseases,[80] arthritis,[50] allergies,[51] cardiac diseases,[53] irritable bowel syndrome,[95] cancer,[18] Urinary tract[71], hyperlipidemia.[61] Among the oral disease, its effect has been studied on dental caries,[55] periodontitis,[41] halitosis,[9] aphthous ulcers,[90] fungal infections,[31] and oral cancer.[98]

Different probiotic strains have been studied for the prevention of dental caries namely: *Lactobacillus rhamnosus* GG,[55] *Lactobacillus rhamnosus* LC 705,[2] *L.reuteri,*[57] *Lactobacillus reuteri* ATCC 55730,[13] *L. rhamnosus* LB21,[83] *L. paracasei* GMNL-33,[17] *Bacillus coagulans,*[36] *Bifidobacterium lactis Bb-12, Lactobacillus acidophilus La-5,*[78] *Lactobacillus acidophilus,* [85] *Lactobacillus rhamnosus hct 70,*[37] *S. salivarius* M18,[9] *Lactobacillus paracasei* SD1,[86] *L. salivarius,*[59] *S. uberis* KJ2, *S. oralis* KJ3, *S. rattus* JH145,[32] *L. reuteri*(DSM 17938 and PTA 5289),[73] *Bifidobacterium lactis,*[60] *Lactobacillus rhamnosus SP1.*[72]

Probiotics has been used with different vehicles such as cheese,[2] milk,[55,83] yoghurt,[57,85] lozenges,[15] capsule,[52] ice creams,[3,78] straw,[13] tablet,[17] gum,[14] powder[36] and drink.[20] Ideally its delivery should be suitable for all ages to receive its benefit. Possemiers et al[69] added probiotics to dark and milk chocolates. It was observed that coating probiotics in chocolates was an excellent solution to protect it from stress conditions and for optimal delivery into the GI tract. Kanafari et al[39] in an invitro experiment proved the effectiveness of probiotic dark chocolate against *S.mutans*. Literature search did not reveal any invivo trials of probiotic chocolates in general as well as in dental research. Considering the growing sales of chocolate, a pragmatic idea would be to manufacture a tooth friendly chocolate.

Hence the aim of this study was to make a customized probiotic chocolate and evaluate its effectiveness in the oral cavity against change in pH and *S. mutans* level.

AIMS

AIMS

- To formulate 3 types (milk, white and dark) of chocolates with probiotics.
- To compare the plaque pH, salivary pH and buffering capacity of the three probiotic chocolates with conventional chocolates in children
- To evaluate the antimicrobial efficacy of the 3 custom made probiotic chocolates against Streptococcus mutans.

REVIEW OF LITERATURE

Nase L, Hatakka K, Savilahti E, Saxelin M, Ponka A, Puossa T, Korpela R, Meurman J H (2001)[55] evaluated the effect of probiotic milk in preventing caries in children aged 1-6 years. A randomized control trial was done on 594 children dividing them into experimental (n=296) and control groups (n=298). The experimental group drank milk containing *Lactobacillus rhamnosus* GG whereas the control group consumed normal milk on weekdays for 7 months. Children's dental caries status was recorded using WHO criteria. Caries risk was assessed through streptococcus mutans colony count with Dentocult SM Strip mutans kit. Results showed a significant reduction in colony count post intervention in the probiotic group and less incidence of dental caries compared to control group. Long term consumption of probiotic milk reduced the incidence of dental caries.

Ahola AJ, Yli-Knuuttila H, Suomalainen T, Poussa T, Ahlstrom A, Meurman JH, Korpela R (2002)[2] evaluated the short-term consumption of cheese containing *Lactobacillus* GG and *Lactobacillus rhamnosus* LC 705 on caries-associated salivary microbial counts in 12- 35 year old adults. The study was a double blind, placebo controlled randomized controlled trial involving 74 subjects. Both the groups ate 15g cheese cubes 5 times a day for 3 weeks. The study group children ate cheese containing *Lactobacillus* GG and *Lactobacillus rhamnosus* LC 705 and the control group children ate normal cheese. Stimulated salivary secretion rates, buffer capacity and counts of salivary *Streptococcus mutans*, yeast and lactobacilli were evaluated. Samples were taken at pre intervention, post intervention and after 3 weeks follow up period. The results showed no statistically significant difference between the groups in *Streptococcus mutans* counts after the intervention, but in the follow up period there was a significantly greater

reduction in these counts in the intervention group compared to the control group. Hence eating cheese in general provides beneficial effect in diminishing caries risk.

Verakaki E, Duggal MS (2003)[94] estimated the acidogenic potential for different European chocolates with varying cocoa content. The test chocolates included diet chocolate (DC), plain European chocolate (PEC 70% cocoa), plain English chocolate (PenC 34% cocoa), milk English chocolate (MenC 20% cocoa), milk European chocolate (MEC 30% cocoa), White chocolate (WC no cocoa), milk chocolate with hazelnuts (MHC 20% cocoa), 10 ml of 10% sucrose and sorbitol solution. Fourteen Participants within 16-50 years consumed 15g of the chocolate. Plaque pH was measured at baseline and at 2, 5, 10, 15, 20 and 30 minutes, after consuming the test chocolates. Diet chocolate was found to have no acidogenic effect on dental plaque. PEC and MHC had a lower acidogenic potential than sucrose.

Nikawa H, Makihira S, Fukushima H, Nishimura H, Ozaki Y, Ishida K, Darmawan M, Hamada T, Hara K, Matsumoto A, Takemoto T, Aimi R (2004)[57] evaluated the inhibitory effect of custom made yogurt containing *Lactobacillus reuteri* against *Streptococcus mutans* along with analyzing the inhibitory effect of various commercially available yogurts. The custom made yogurt and a commercially available yogurt containing *Lactobacillus reuteri* showed a significant inhibitory effect on the growth of S. mutans, whilst yogurt with other lactobacilli species did not show such inhibition. Further a double-blind, placebo-controlled trial was conducted including 40 subjects aged 20 years for a test period of 2 weeks. The study subjects were given 95 g of either a placebo yogurt containing *L.bulgaris and S. thermophilus* or yogurt containing *L.reuteri,* during their lunch period. The saliva samples were inncoluated in Mitis salivarus agar and incubated at 37°C for 48 hours, to determine the colony-forming unit of mutans streptococci. Results revealed that consuming yogurt with *L. reuteri* significantly

reduced the levels of mutans streptococci, than the placebo yogurt. *L. reuteri* combined with yogurt was found to be effective in reducing the caries risk.

Montalto M, Vastola M, Marigo L, Covino M, Graziosetto R, Curigliano V, Santoro L, Cuoco L, Manna R,Gasbarrini G (2004)[52] evaluated the change in lactobacillus and streptococcus colony count after probiotic usage through the oral route. Thirty-five healthy volunteers were randomized into 3 groups; group A (n = 14) received probiotics in capsules and placebo in liquid form; group B (n = 16) took liquid probiotics and placebo in capsules, and group C (n = 5) used placebo in both liquid and capsule form. The salivary counts of *lactobacilli* species and *S. mutans* were measured using the CRT® bacteria kit. Results showed a significant improvement in *lactobacillus sp.* colony count in the saliva with both capsules and gels. There was no significant difference in *streptococcus* colony counts. The increase in lactobacilli count with inhibitory effect on other bacteria could be a treatment option in the long-term prevention of caries.

Caglar E, Cildir SK, Ergeneli S, Sandalli N, Twetman S (2006)[13] investigated the effect of the *Lactobacillus reuteri* ATCC 55730 on the levels of salivary mutans streptococci and lactobacilli. A parallel arm placebo controlled trial was conducted on 120 adults aged 20-24 years for 3 weeks. Group A ingested 200 ml of water through a prepared straw containing *L. reuteri* ATCC 55730, group B drank 200 ml water through a placebo straw. Group C was given one tablet containing *L. reuteri* ATCC 55730 and group D received placebo tablets without bacteria. Salivary mutans streptococci and lactobacilli were enumerated with Dentocult SM and Dentocult LB chair-side kits at baseline and 1 day after the final ingestion. There was a statistically significant reduction in *S.mutans* colony count with straw and tablets. Lactobacillus

counts were not statistically significant. Both modes of deliveries probiotic were effective in reducing the Streptococcus mutans colony count.

Caglar E, Kavaloglu SC, Kuscu OO, Sandalli N, Holgerson PL, Twetman S (2007)[14] carried out a randomized controlled trial to evaluate the effectiveness of probiotic and xylitol against mutans streptococcus (MS) and lactobaccili (LB). About 80 children aged 21-24 years were randomly assigned to one of the 4 groups: group A- probiotic gum; group B- xylitol gum; group C- probiotic and xylitol gum and group D- placebo gum. The probiotic gum contained *Lactobacilli reuteri* ATCC 55730 and ATCC PTA 5289. Children received the gums for a period of 3 weeks. Baseline saliva and 1 day post intervention saliva was collected and tested for MS and LB with chair-side kits. A statistically significant reduction of salivary MS was seen in group A and B after the intervention when compared with baseline. Group C also showed reduction in colony count which was not statistically significant. There was no change in the colony counts in group D. They concluded that use of probiotic and xylitol chewing gums showed reduction in mutans streptococcus but they were ineffective when used in combination.

Caglar E, Kuscu OO, Cildir SK, Kuvvetli SS, Sandalli N (2008)[15] investigated the effect of *Lactobacillus reuteri* on salivary *Streptococcus mutans* colony count. A pacifier like medical device was used to deliver the probiotic lozenges. Twenty adults were randomly divided into experimental and control group with 10 in each group. The experimental group sucked probiotic lozenge with *L. reuteri* (ATCC 55730) and *L. reuteri* (ATCC PTA 5289), once daily for 10 days. While the control subjects received placebo medical devices. Salivary mutans streptococci and lactobacilli were evaluated with chair-side kits at baseline and 1 day after the final ingestion. Salivary *S. mutans* levels in the experimental group significantly reduced. Lozenges were shown to be an effective means of probiotic delivery.

Stecksen-Blicks C, Sjostrom I, Twetman S (2009)[83] combined *L. rhamnosus* LB21 and fluoride in milk to conduct an invivo trial in 248 preschool children aged 1 to 5 years. The primary aim of the study was to determine the caries-preventive effect while the secondary aim remained at evaluating infections, use of antibiotics and medical care. Children in the intervention group received milk containing fluoride of 2.5 mg/litre and *L. rhamnosus* LB21. Control group received medium fat milk. Both the groups were provided with the milk, once daily for 21 months. After the intervention the mean caries experience measured with WHO criteria was lesser in the intervention group than the control group. The intervention group had 60% fewer days with antibiotic therapy and 50% less days with otitis media. The study showed the anti caries effect as well as the general health benefits of long term consumption of probiotics.

Hegde AM, Shetty R, Sequeira AR (2009)[34] evaluated the acidogenicity of 6 chocolates available in Indian market, among 30 dental volunteers. Six commercially available chocolates were divided into two subgroups. Unfilled group contained plain milk, dark and diet chocolates whereas the filled group had fruits and nuts, caramel and coconut chocolates. Plaque pH was measured at baseline and at 5, 10, 15, 20, and 30 minutes after consumption of chocolates. In unfilled group, milk chocolate had maximum pH drop at 20 minutes and diet chocolate had minimum pH drop at 10 minutes. Fruit and nut had maximum pH drop at 20 minutes and caramel had minimum pH drop at 15 minutes. Filled chocolates were found to be more acidogenic than unfilled chocolates.

Chuang LC, Huang CS, Ou-Yang LW, Lin SY (2011)[17] conducted a double-blinded, randomized, placebo-controlled study with 78 adults aged 20-26 years for 2 weeks. The subjects received a oral tablet (1g) containing either *L. paracasei* GMNL-33 or placebo tablet thrice daily.

Dentocult SM Strip kit was used to check the bacterial counts of salivary *S. mutans*, Dentocult LB Dip Slide for *lactobacillus* and Dentobuff Strip for salivary buffering capacity of saliva. The measurements were done at the baseline (T1), the completion (T2) of study period and 2 weeks post intervention (T3). Though not significant, a reduction in the salivary S. mutans count was detected between T2 and T3 of the intervention group. There was no significant difference in the lactobacillus count and buffering capacity of saliva. Short term consumption of probiotic tablet reduced the risk for caries.

Jindal G, Pandey RK, Agarwal J, Singh M (2011)[36] conducted a study with 150 children aged 7-14 years. The children were divided into 3 groups; group A received placebo powder, group B received freeze drided *Lactobacillus rhamnosus* and *Bifidobacterium* while group C received *Bacillus coagulans*. The children were instructed to mix the preparation with 20ml of water to swish and swallow for 14 days. Mutans streptococcus colony count was checked on mitis salivarius bacitracin agar at baseline and 14 days post intervention. There was a significant reduction of colony count in both group B and C at 14 days post intervention. Dietary supplements with probiotics could be a cheap alternative to reduce the incidence of dental caries.

Singh RP, Damle SG, Chawla A (2011)[78] conducted a study with ice cream containing *Bifidobacterium lactis Bb-12 and Lactobacillus acidophilus La-5*. It was a double blind, placebo controlled study which included 40 children aged 12-14 years. Children were given ice cream weighing 52 g for 10 days after which there was a wash out period of 2 weeks. In the next phase, the ice creams were inter changed between groups. The *S.mutans* colony count was measured using Dentocult SM at baseline and post intervention. There was significant reduction in the colony count post consumption of probiotic ice cream compared to baseline in both the groups. Ice creams can serve as a vehicle for delivering probiotics effectively.

Sudhir R, Praveen P, Anantharaj A, Venkataraghavan K (2012)[85] compared the effect of probiotic curd containing *Lactobacillus acidophilus* vs normal curd consumption in children. Forty children were randomly divided into 2 groups for an interventional period of 30 days. Baseline and post intervention saliva samples were collected and cultured in Mitis salivarius bacitracin agar to estimate the *S.mutans* colony count. There was a significant reduction in the colony count after 30 days of probiotic curd consumption. Short‑term consumption of probiotic curds can reduce oral *S. Mutans* counts.

Juneja A, Kakade A (2012)[37] conducted a study to determine the effectiveness of short time probiotic intake on 40 children aged 12-15 years for 9 weeks. The study was divided into 3 phases of 3 weeks each. First phase was baseline, while in the second phase children were given either milk containing *Lactobacillus rhamnosus hct 70 or* placebo milk. The third phase was follow up. After each phase salivary samples were collected. The intervention group had a statistically significant reduction of *S.mutans* counts at post intervention and follow up period. The study suggested a protective and preventive role of probiotic *Lactobacillus rhamnosus hct 70* against dental caries.

Khanafari A, Hosseini Porgham S, Tajabadi Ebrahimi M (2012)[39] evaluated the inhibitory potential of *S. mutans* using three probiotic strains *Lactobaccilus plantarum, L. rhamnosus and L. acidophilus.* The antimicrobial effect of the probiotic strain against *S.mutans* was determined by deferred cross streak method and susceptibility was tested by disk diffusion method, in which *L. plantarum* was identified to be having the maximal antimicrobial effect. Probiotic strains were then added to dark chocolate at 10^8CFU/mL and their antimicrobial effect against *S.mutans* was evaluated using disk diffusion susceptibility test. Probiotic chocolate

containing *L.plantarum* showed the maximal inhibitory zone. Chocolates can be used as a means for delivering probiotics.

Dhawan R, Dhawan S (2013)[21] assessed the effectiveness of commercially available probiotic on plaque, gingivitis, and salivary *Streptococcus mutans* levels in subjects with chronic gingivitis. Commercially available probiotic BIFILAC- HP capsule containing *Lactobacillus sporogenes, Streptococcus faecalis T-110JPC, Clostridium butyrium TO-A, and Bacillus mesentericus TO-A JPC* was used. The experimental group (n=17) probiotic capsule and the control group was given placebo capsule to swallow for a period of 2 weeks. Plaque index, gingival index and streptococcus mutans colony count was measured at baseline, post intervention and 2 weeks post intervention. Probiotic group showed a statistically significant difference from the placebo group in all the parameters assessed, throughout the study period. The use of probiotics could decrease the disease processes in oral cavity effectively.

Chinnappa A, Konde H, Konde S, Raj S, Beena JP (2013)[16] conducted a clinical trial to compare *Streptococcus mutans* count after consuming probiotic curd or ice cream. Forty children aged 12-14 years were randomly divided into probiotic ice cream, plain ice cream, probiotic yogurt or plain yogurt group. All the groups received 100ml of the product for 7 days. Saliva samples were assessed at baseline, 1 hour and 7 days post intervention using Mitis salivarius Bacitracin agar. There was a reduction in colony count at 1 hour post intervention for all the groups. Reduction in colony count was significant in the probiotic groups at 7 days compared to baseline. However there was no significant difference between probiotic yogurt and ice cream at 1 hour and 7 days. The use of probiotic products can be an alternative strategy for displacing pathogenic micro organisms.

Burton JP, Drummond BK, Chilcott CN, Tagg JR, Thomson WM, Hale JD, Wescombe PA (2013)[9] evaluated the effect of probiotic lozenges on plaque, gingival health and oral microflora. Children aged 5- 10 year were divided into probiotic group (n=40) and placebo group (n=43). Probiotic group received 2 lozenges per day containing *S. salivarius* M18 for 3 months and the placebo group received similar lozenges without probiotic. Plaque, gingival health assessment and salivary assessments were done at 1, 3 and 7 months. Chromogenic agar for *Candida spp,* Rogosa SL agar for lactobacilli, Mitis Salivarius agar for *S. salivarius* and Tryptone Yeast Cystine Sucrose Bacitracin (TYCSB) agar for *S.mutans* was used. Gingival health and *S.salivarius* colony count improved while *S.mutans* colony count reduced in the probiotic group. S. salivarius M18 can provide oral health benefits on long term consumption.

Taipale T, Pienihakkinen K, Alanen P, Jokela J, Soderling E (2013)[86] evaluated the effect of *Lactobacillus paracasei* SD1 on the number of salivary mutans streptococci, lactobacilli, and yeasts. The study consisted of 40 adults randomly divided into probiotic milk and standard milk group. The probiotic group received milk containing *Lactobacillus paracasei* SD1 and the control group received standard milk once daily for 4 weeks. Salivary samples were collected at baseline and once a week for 4 weeks post intervention. The colony count of salivary mutans streptococci, lactobacilli, and yeasts were evaluated using mitis salivarius bacitracin agar, MRS, and Sabouraud Dextrose agar respectively. A statistically significant reduction in mutans streptococci count occurred in probiotic group compared to the baseline. A significant increase in lactobacilli count also occurred and was detected up to 4 weeks post intervention. No significant reduction in yeast count was observed. Short term daily intake of *Lactobacillus paracasei* SD1 reduced the *S.mutans* colony count.

Nishihara T, Suzuki N, Yoneda M, Hirofuji T (2014)[59] evaluated the effectiveness of *L. salivarius* containing tablets on caries risk factors. Sixty four adults were divided into four groups; *L. salivarius* WB21 (n=17), *L. salivarius* TI 2711 (n=16), Ovalgen® DC (N=13) (IgY antibody) and xylitol tablet (N=18) group. The participants were instructed to place the tablet on the tongue and then allow it to dissolve. Levels of mutans streptococci and lactobacilli, salivary flow, salivary pH, and salivary buffering capacity were assessed before and after taking the tablets. The level of mutans streptococci was evaluated using Dentocult SM Strip mutans kit. Further a short term trial was conducted with *L. salivarius* WB21- containing tablets. Participants were given 3 tablets a day for 2 weeks. The levels of mutans streptococci decreased in the *L. salivarius* WB21, TI 2711 and Ovalgen® DC groups compared to the xylitol group. Lactobacilli levels significantly increased in the *L. salivarius* WB21 and TI 2711 groups. Salivary flow and salivary pH did not differ significantly between the groups. The salivary buffering capacity significantly increased in the L. salivarius TI 2711 group and Ovalgen® DC group than the xylitol group. The short-term administration trial showed that the *L. salivarius* WB21-containing tablets significantly decreased the number of mutans streptococci and therby increases the resistance to caries risk.

Hedayati-Hajikand T, Lundberg U, Eldh C, Twetman S (2015)[32] assessed the effect of chewing probiotic tablet in developing early childhood caries among 2-3 year old children for a period of 1 year. The study included 138 children randomly divided into probiotic and placebo group. The probiotic group received one chewing tablet per day containing *S. uberis* KJ2, *S. oralis* KJ3, *S. rattus* JH145 while the placebo group received tablet without probiotic. Decrease in caries increment was found in the probiotic group compared to the placebo group. Results

suggested that early childhood caries development could be reduced through administration of probiotic tablets.

Romani Vestman N, Chen T, Lif Holgerson P, Ohman C, Johansson I (2015)[73] conducted a randomized controlled trial to evaluate the effects of *L. reuteri*(DSM 17938 and PTA 5289) on the oral microbiota composition using 454 pyrosequencing and the HOMD along with *L. reuteri* specific culturing and PCR detection. Forty adults were randomly divided into probiotic lozenges and placebo group, receiving 2 lozenges per day for 12 weeks. Salivary samples were collected at baseline, 4, 8 and 12 weeks during intervention. Follow-up samples after intervention were collected 1 and 6 months. Streptococcus was the most common genus and the *S. oralis/ S. mitis/S. mitis bv2/S. infantis* group comprised the dominant species. There was shift in microbiotia with reduced *S. mutans, S. anginosus, N. mucosa, Fusobacterium periodicum, F. nucleatum ss vincentii,* and *Prevotella maculosa* detection. This shift was observed upto 1 month post intervention. *L.reuteri* (DSM 17938 and PTA 5289) was effective in reducing the pathogenic microflora.

Nozari A, Motamedifar M, Seifi N, Hatamizargaran Z, Ranjbar MA (2015)[60] conducted a randomized controlled trial to assess the effectiveness of probiotic yogurt in children aged 6-12 years. The children were randomly divided into case (n=25) and control (n=24) groups. The case group received probiotic yogurt containing *Bifidobacterium lactis* (1×106 per gram) and the control group received normal yogurt for 2 weeks. A wash out period of 2 weeks was given and again the children were given yogurt for 2 weeks. Pre treatment, between regimen and post treatment saliva was collected. *Streptococcus mutans* colony counting was done on blood agar and lactobacillus colony count was done on tomato juice agar. Results did not show a statistically significant difference in the colony counts in the probiotic as well as control group.

The short term consumption of probiotic yogurt did not reduce the *streptococcus mutans* and *lactobacilli* colony count.

Ashwin D, Vijayaprasad KE , Taranath M, Ramagoni NK, Naras A, Sarpangala M (2015)[3] conducted a double blind randomized controlled trial with 60 children of age 6 to 12 years. Probiotic ice cream containing *Bifidobacterium lactis* Bb-12 and *Lactobacillus acidophilus* La-5 was given to the children for 7 consecutive days. *Streptococcus mutans* colony count was assessed at baseline, 7 days, 30 days and six months using Dentocult SM kit. There was a reduction in the colony count at 7 and 30 days but the colony count was similar to baseline after 6 months.

Mahantesha T, Parveen KM, Praveen NH, Asha N, Ashwin D, Vinutna B (2015)[46] conducted a randomized controlled trial including 50 children of 6 – 12 years with zero DMFT. Children were randomly divided into either probiotic ice cream group or probiotic milk group. Children were given the probiotic milk for 7 consecutive days. Salivary samples were collected at baseline, one day and 3 months post intervention. The samples were assessed for *S.mutans* colony count. Both the probiotic groups showed a statistically significant reduction in colony count at 1 day post intervention period. The probiotic ice cream showed a reduction in colony count after 3 months compared to the baseline. But the probiotic milk group did not show a significant reduction in colony count. Ice cream was as a better vehicle for probiotic delivery compared to probiotic milk.

Deo PN, Deshmukh R (2015)[20] evaluated the efficacy of probiotic drink containing *Lactobacilli casei* on salivary *Streptococcus mutans* count. The study population was 50 adults with 18-35 years of age. The study population was given 65ml of the probiotic drink once daily for 7 days. Baseline and post intervention *S.mutans* colony counting was done on Mitis salivarius

bacitracin agar from saliva sample. There was a significant reduction in the colony counts between baseline and post intervention. Probiotics have a promising role to play in preventing dental caries.

Yousuf A, Nagaraj A, Ganta S, Sidiq M, Pareek S, Vishnani P, Acharya S, Singh K (2015)[97] conducted a trail in 33 children of 12-15 year old. The children were randomly divided into 3 groups with 11 in each group. They either received a freeze dried preparation of *Lactobacillus acidophilus, Bifidobacterium longum, Bifidobacterium bifidum* and *Bifidobacterium lactis* or lactic acid bacillus only. The control group received placebo powder. Saliva samples were collected at baseline, 7, 14 and 21 days and *S.muatns* colony count was done in Mitis salivarius bacitracin agar. Both the probiotic groups showed a reduced colony count after the second week. It was concluded that oral probiotics showed a short term effect in reducing *S. mutans.*

Bhalla M, Ingle NA, Kaur N, Yadav P (2015)[6] evaluated the effect of probiotic curd on mutans streptococci levels in saliva. Children aged 12-14 years were randomly divided into probiotic curd (n=15) and plain curd (n=15) group. The probiotic curd group was supplied with curd containing *B. lactis* 12. Both the groups were provided with 200ml of curd for 7 days. Salivary mutans streptococcus level was estimated at baseline, at 1 hour and on the 7^{th} day by using mitis Salivarius Bacitracin Agar. The colony counts reduced in the probiotic group at 1 hour and 7^{th} day compared to baseline values. There was a statistically significant reduction in the colony count between the groups at 1 hour and 7^{th} day.

Vasanthakumar H, Sharan J, D.Cruz AM (2016)[93] evaluated the acidogenecity of 5 different types of chocolates over 10 adults aged 20-30 years. The chocolates used were white chocolate, milk chocolate, dark chocolate, caramel chocolate and 10% sucrose solution. Plaque

pH was estimated at 10, 20, 30 and 45 minutes using pH test strips. Caramel chocolate had the maximum decrease in plaque pH at 20 minutes after consumption. The least drop in pH was noted for dark chocolate. At the end of 45 minutes, the dental retention measured by sugar clearance was highest for the caramel chocolate. Dark chocolates have a greater content of cocoa and less sugar making it the least cariogenic.

Nirmala S, Quadar MA, Veluru S (2016)[58] compared the acidogenecity of 6 different types of chocolates dividing them into unfilled (dark and milk chocolate), filled (wafer and fruit and nuts chocolate), and candy (hard milk and mango-flavored candy) groups. Plaque pH values and salivary sugar clearance rates were assessed at baseline, 5, 10, 15, 20 and 30 mins after consumption. Dark chocolate had a high fall in pH and milk chocolate had low salivary sugar clearance which signifies that unfilled chocolates are more cariogenic than other chocolates.

Rodriguez G, Ruiz B, Faleiros S, Vistoso A, Marró ML, Sanchez J, Urzua I, Cabello R (2016)[72] conducted a randomized controlled study in 2-3 year old children with the aim of a comparing probiotic milk with standard milk. Two hundred and sixty one children were divided into experimental and control group. Experimental group (n=150) were given 150ml of milk supplemented with *Lactobacillus rhamnosus SP1* whereas the placebo group (n=111) was given standard milk. Microbiological samples of the milk were done to confirm the presence of probiotic bacteria. Children were supplemented with the milk during weekdays for 10 months. Clinical examinations were done following International Caries Detection and Assessment System ICDAS criteria at the baseline and end of the study. Results showed a decline in caries increment at the end of the study in the probiotic group. Regular intake of probiotic milk reduced the risk for dental caries in preschool children.

Srivastava S, Saha S, Kumari M, Mohd S (2016)[81] estimated the role of probiotic curd on salivary Streptococcus mutans count after 7 days intervention. Sixty adults aged 20-25 years were randomly divided into probiotic and plain curd groups, with 30 in each group. Participants were supplied with 100ml of curd each day for 7 days. Salivary samples were collected at baseline, half an hour, 1 hour and 7 days after intervention. pH was measured using pH meter and Mitis Salivarius Bacitracin agar to estimate *S. mutans* count. Results showed a reduction in salivary pH after ½ hour and 1 hour in both the groups but after 7 days, probiotic curd showed a statistically significant increase in salivary pH. Probiotic curd showed statistically significant reduction in S. mutans colony counts compared to normal curd.

Ghasemi E, Mazaheri R, Tahmourespour A (2017)[29] investigated the effect of probiotic yogurt vs xylitol chewing gum on salivary *streptococcus mutans* colony count. Fifty adults were randomly divided into two groups. Probiotic group received 200g yogurt containing *Lactobacillus acidophilus and Bifidobacterium bifidum* once daily for 3 weeks. Xylitol group received 2 chewing gums (xylitol content: 5.58 grams daily) 3 times daily after each meal for 3 weeks. Saliva samples were collected at baseline, 1 day, 2 weeks and 4 weeks after the intervention. Samples were cultured on mitis salivarius bacitracin agar and salivary *S. mutans* counts were determined. Both the groups showed a reduction in *S.mutans* colony count at all the time periods of investigation compared to baseline. Though probiotic group showed a higher reduction in *S.mutans* count when compared to xylitol group, it was not statistically significant. Long term consumption of probiotics and xylitol has caries preventive effect.

METHODOLOGY

The present randomized controlled trial was conducted in the Department of Pediatric and Preventive Dentistry, K S R Institute of Dental Science and Research (KSRIDSR). The study was planned and organized in association with various schools in Tiruchengode to determine the plaque pH, salivary pH and buffering capacity after consumption of custom made probiotic chocolates in children. The study design and protocol was analyzed and approved by the Institutional Review Board and Institutional Ethics Committee of KSRIDSR, Tiruchengode, Tamilnadu. The purpose of the study was explained to the school authorities and their approval was obtained. A written consent in mother tongue (Tamil) was also obtained from the parents of the children who participated in the study.

Materials for preparation of chocolates

- Bifilac^{TM} sachet (Tablets India Ltd, Chennai, India)
- Dairy milk (Cadburys, Mumbai ,India)
- Milky bar (Nestle, Gurgaon, India)
- Dark chocolate (Amul, Gujarat, India)
- Sterile bowl and spatula
- Chocolate molds
- Aluminium foil
- Refrigerator

Materials for determining plaque pH salivary pH and buffering capacity

- Diagnostic instrument set – consisting of a mouth mirror, explorer and tweezer in kidney tray.
- Disposable mask

- Disposable gloves
- Paraffin wax
- Sterile sample container (uricups)
- 3.0 ml disposable pipette
- 5.0 ml glass measuring pipette
- 5.0 ml disposable test tubes with caps
- Octanol solution 500ml (Himedia, All India laboratories)
- Hannah pH meter (Oakton, pH tester 20, Singapore)
- Neutral pH 4 and pH 7 tablets (Merck, Mumbai)
- Sterile explorer
- Distilled water
- 0.005 mol/litre HCl

Materials for microbial colony count

- Thioglycollate agar
- Bacitracin disk 10 units
- Blood
- Hot water bath
- Conical flask 2000ml
- 1000 µl pipette
- Disposable micro tips
- Petri plates
- Glass rod

- Ethanol
- Plate rotator
- Laminar flow chamber
- Incubator
- Automatic colony counter

Inclusion criteria

- School children of 8-12 years.
- def/DMFT score ≤ 3

The exclusion criteria includes children

- On antibiotics
- Under probiotics
- Using xylitol chewing gums
- Allergic to dairy products
- Medically compromised like autoimmune disorders.

Invitro study

Determining the minimum inhibitory concentration

. The probiotic chosen for the present study was Bifilac^{TM} sachet which contains:

Prebiotics: *Streptococcus faecalis* T-110 (30 million)

Clostridium butyricum TO-A (2 million)

Bacillus mesentricus TO-A (1 million)

Probiotic: *Lactobacillus sporogenes* (50 million)

It was used for antimicrobial tests against *S.mutans*. The minimum inhibitory concentration (MIC) of the probiotic against *S.mutans* was determined to be 0.5µg/mL.

Preparation of probiotic chocolate

Commercially available chocolate bars of white chocolate (Milkybar), milk chocolate (Dairy milk) and dark chocolate (Amul) were used. About 13g of the chocolates were melted at 36–37ºC and the molten chocolates were tempered at 34ºC for 10 minutes. The MIC concentration of probiotic with 10^8 CFU (according to 0.4 McFarland units) was added to the 13g molten chocolates and thoroughly mixed with a stirrer. The chocolates were then poured in a mould and set for cooling at 4ºC. The hardened chocolates were wrapped in aluminum foil and they were stored in refrigerator.

Antimicrobial effect of probiotic Chocolate on Streptococcus mutans

The antimicrobial effect of the probiotic chocolate was evaluated by agar well diffusion method. *Streptococcus mutans* ATCC 25175 was lawn cultured in Muller Hinton agar following which 6mm sterile cork broker was used to punch holes in agar plates. Each plate had 2 holes, one for the normal chocolate and the other for the probiotic version of the same chocolate. Triplicates of plates were done and incubated at 37ºC for 24- 48 hours to check for the zone of inhibition. Zone of inhibitions were present for all the 3 probiotic chocolates confirming the viability of probiotics after adding to chocolates.

Invivo study

Part 1

This randomized controlled trial was carried out by screening 132 children of aged 8 to 12 years. Ninety children who fulfilled the inclusion and exclusion criteria were selected. A

blinded investigator randomly divided the children into 3 groups with 30 children in each group using table of random numbers. Primary investigator was not aware of the allocation process. The study was conducted in 2 phases. For phase 1 of the trial the groups were:

- Milk chocolate (MC)
- White chocolate (WC)
- Dark chocolate (DC)

For phase 2 of the trial the groups were:

- Probiotic Milk chocolate (PMC)
- Probiotic White chocolate (PWC)
- Probiotic Dark chocolate (PDC)

Saliva and plaque collection

Children in all the groups were asked not to brush their teeth for 48 hours before the day of sample collection. This ensured the presence of old plaque which would contain representative oral bacterial flora. On the test day, they were refrained from eating or drinking 2 hours before sample collection. All the salivary samples were collected at 11 am to reduce any circadian variation in the salivary flow. Children chewed paraffin wax for the collection of baseline salivary samples. The children were provided with sterile uricups to expectorate saliva into the container. Saliva was collected in drool method until 2 ml of saliva was collected.

The plaque samples were collected from pooled plaque. Approximately 1mg was removed from six buccal surfaces of posterior teeth representing all the quadrants of the mouth. Sterilized tooth pick was used for the plaque collection. Forsdicks (1957) method modified by

Rugg-Gunn(1975) was used for estimation of plaque pH. Each plaque sample was thoroughly mixed with 20 ml of distilled water, measured by a pipette. The samples were thoroughly mixed until it dissolved. The plaque and salivary samples were further tested for pH and buffering capacity.

Determination of plaque pH, salivary pH and buffering capacity

The plaque and salivary pH were measured by two blinded examiners using Hannah pH meter. The head of pH bulb was completely immersed into the sample for each of the salivary and plaque samples. The values displayed digitally were recorded after the fluctuations in the reading stopped. The electrode was cleaned with a stream of distilled water between each measurement.

The salivary buffering capacity was determined by the classical Ericson's test (1959). For stimulated saliva 0.005 mol per L of hydrochloric acid (HCl) was used. To prepare 1000ml of a 0.005 mol per L of HCl, 0.14 ml of 37.2% HCl was added with distilled water. The HCl was collected by using micropipette to obtain accurate volume for preparation. For measuring the buffering capacity, 1.0ml of the saliva was transferred to 3.0 ml of 0.005 mol per L HCl. One drop of 2-Octanol was added to prevent the foaming reaction and the samples were mixed for 20 minutes to remove carbon dioxide. Buffering capacity was evaluated electrometrically using pH meter. After recording the baseline plaque pH and salivary pH, children were provided with their respective chocolates. Since the chocolates were wrapped in similar aluminum foil, children were blinded of the group they belonged. All the children were instructed to eat the chocolates for 5 minutes to generalize the consumption time. Children were monitored to consume the chocolate completely without sharing. The saliva and plaque samples were collected and

assessed for salivary pH, plaque pH and buffering capacity at 10 minutes, 30 minutes and 60 minutes post consumption of the chocolates.

Phase 2 of the trail was done after a wash out period of 20 days. The children assigned to the milk, white and dark chocolates were assigned to their respective probiotic groups. Similar methodology was followed in the estimation of the plaque pH, salivary pH and buffering capacity. The results were tabulated and analyzed statistically.

Part 2

Determination of *S.mutans* colony count*:*

For the second part of the invivo trial, children were recruited following the inclusion and exclusion criteria. Children were randomly divided into 3 groups with 20 in each group using table of random numbers. Primary investigator was not aware of the allocation process. The groups were:

Group I - Probiotic dairy milk

Group II - Probiotic milky bar and

Group III - Probiotic dark chocolate

Baseline unstimulated salivary samples were collected at 11 am using sterile uricups. Children were monitored to consume the chocolate completely without sharing. Children were provided with probiotic chocolates for 5 consecutive days in a week at their morning recess break at 11am. Children were instructed to maintain oral hygiene throughout the study period. Saliva samples were collected on the post intervention day 2 hours after consumption of the

chocolates. Salivary samples were again collected at 2 weeks and 4 weeks post intervention. *S.mutans* count was evaluated using spread plate method.

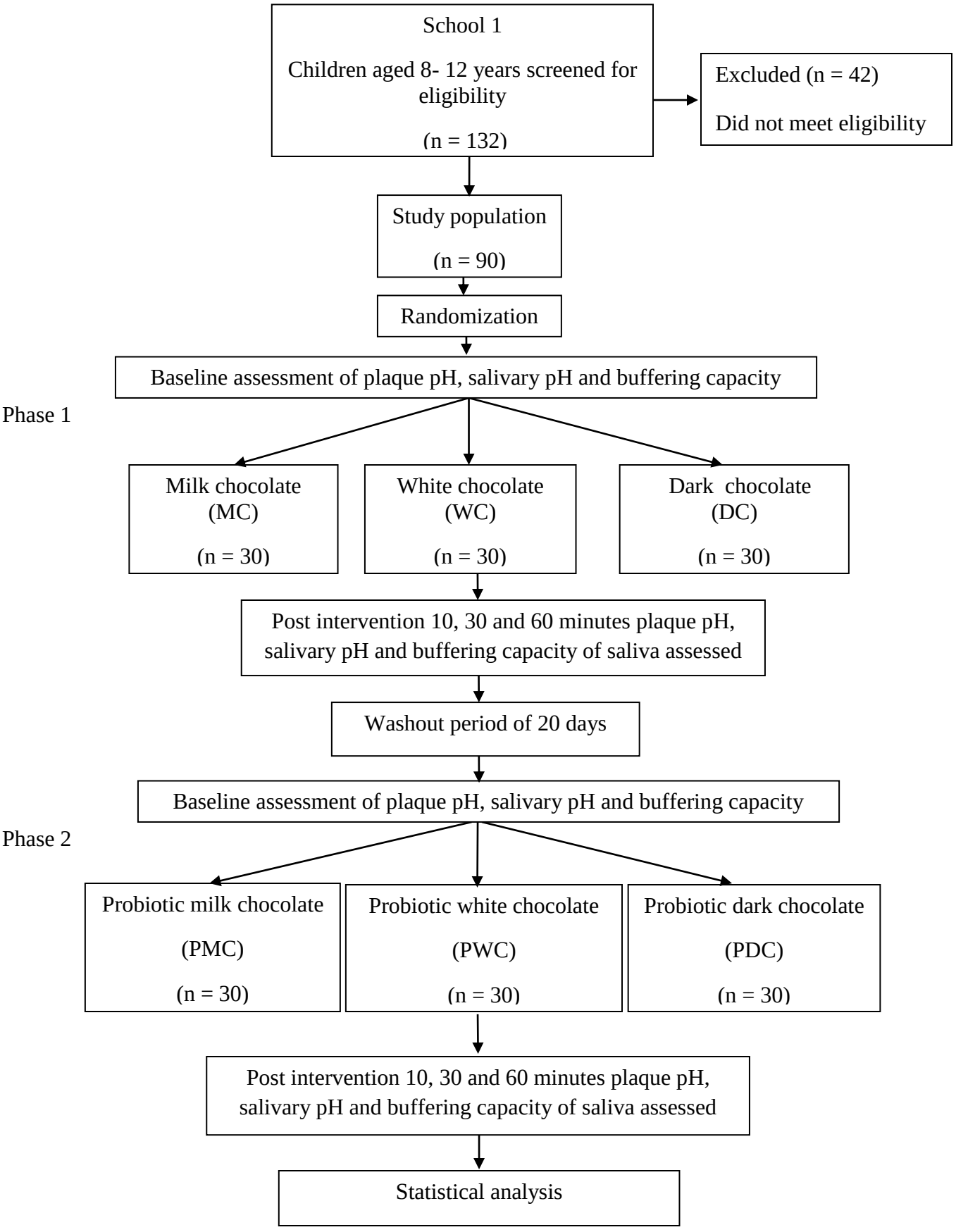
. Thioglycolate sucrose blood bacitarcin agar (TSBB) was used for culturing *S. mutans*. For preparation of this selective media, trypticase soy agar 4g, Yeast extracts 1g, Thioglycolate 3g, Sucrose 20g, distilled water 100 ml was used. The medium was autoclaved at 121°C, 15 lbs pressure for 15 mins and then it was cooled at 55°C. Two disk of bacitracin (each contains 10 Units) was added and kept in a water bath to maintain the temperature. Finally 2 ml of blood was added to it and mixed.

Molten cooled agar (approx. 15mL) was then poured into the petri dish. Saliva samples of about 1 ml were serially diluted 3 times upto 10^{-3} dilution. 1ml of diluted saliva was added in the center of sterile Petri dish using a sterile pipette. After the solidification of the agar, 1 ml of the diluted saliva was plated by using a bent glass rod on the agar media and the plates were inverted and incubated at 37°C for 48 to 72 hours. The colonies were identified by morphology and confirmed using gram staining and catalase test. The results were tabulated and analyzed statistically.

Statistical analysis:

Statistical analysis was done using IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. The statistical significance was set at $p \leq 0.05$. Intragroup comparison was done using paired t Test. Analysis of variance (ANOVA) test was employed to analyse the change in the mean values for intergroup comparison.

Consort flowchart - Trial 1



Consort flow chart –Trial 2

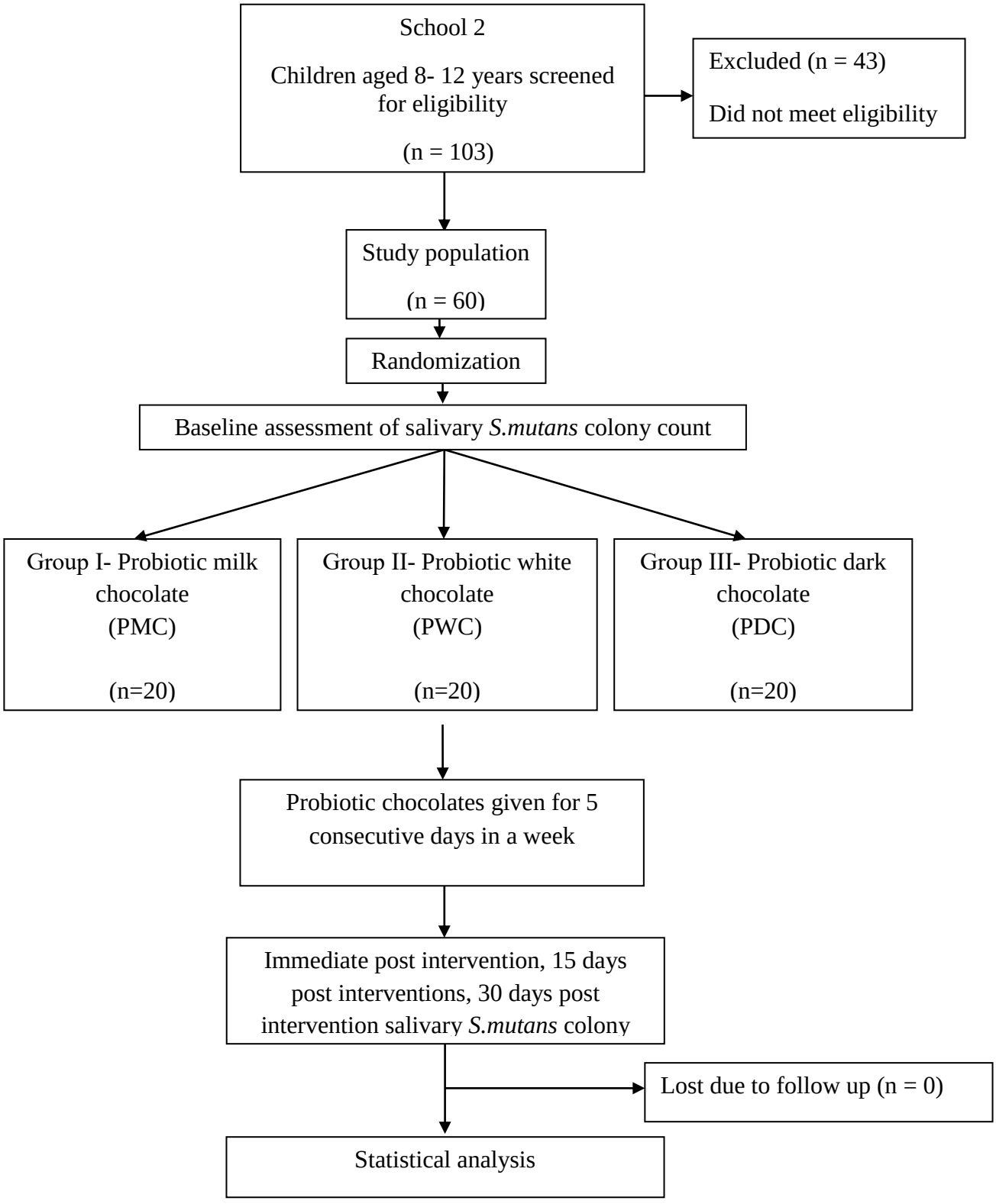


Figure 1. Armamentarium

Figure 2. Probiotic used for chocolate preparation

Figure 3.Probiotic chocolates

Figure 4. Saliva sample collection

Figure 5. Plaque collection using sterile tooth pick

Figure 6. Distribution of chocolates

Figure 8. Samples collected

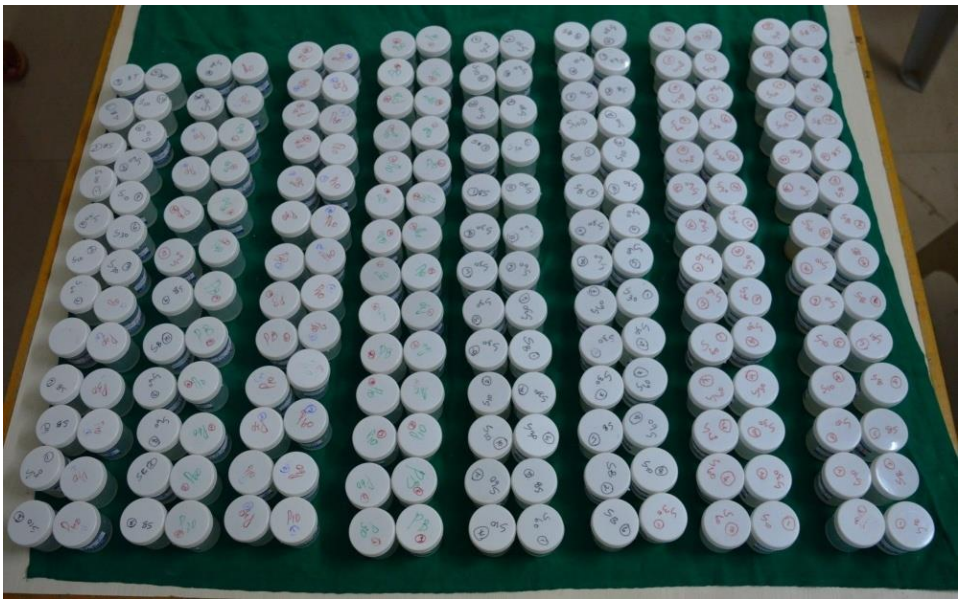
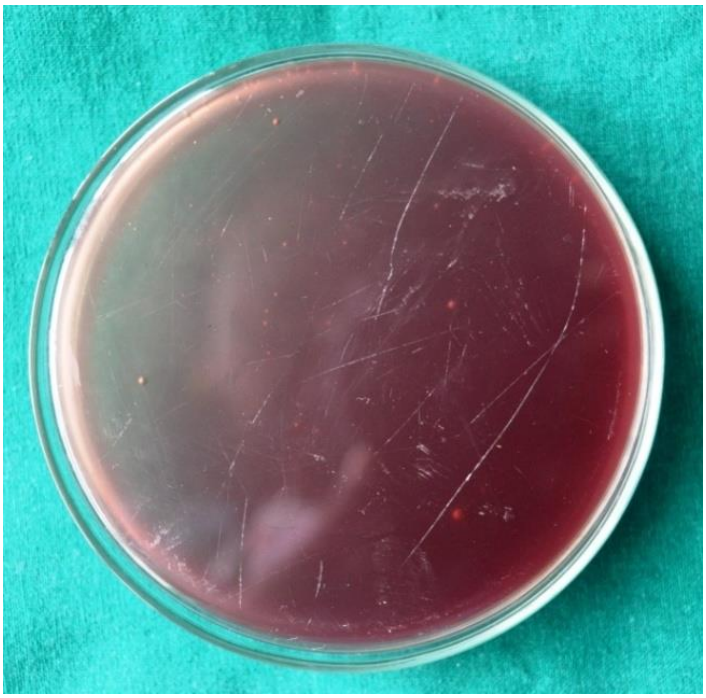


Figure 7. Selective media

Figure 8. Preparation of selective media

Figure 9. Prepared agar plate



RESULTS

Table 1. Results for invitro comparison of the six chocolate groups against *S.mutans*

Groups	Zone of inhibition (mm) Mean ± SD
MC	No zone observed
WC	3.15±0.54
DC	7.12 ± 0.11
PMC	8.94 ± 0.15
PWC	9.02 ± 0.17
PDC	11.82 ± 0.40

Table 1 shows the results for invitro comparison of the six chocolate groups against *S.mutans*. Milk chocolate showed no zone of inhibition against *S.mutans*. Addition of probiotic to milk chocolate produced zone of inhibition. White chocolate showed inhibition zone which was lesser than probiotic white chocolate. Dark chocolate showed the maximum zone of inhibition among the normal chocolate groups. Probiotic dark chocolate showed the maximum zone of inhibition among all the chocolate groups studied.

Table 2. Mean distribution of plaque pH in six chocolate groups

Time	MC	WC	DC	PMC	PWC	PDC
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
Baseline	6.17±0.27	6.51±0.19	6.52±0.27	6.63±0.20	6.57±0.36	6.57±0.26
10 minutes	5.28±0.45	5.76±0.48	6.06±0.47	5.87±0.46	6.15±0.31	5.82±0.54
30 minutes	5.86±0.23	6.25±0.21	6.31±0.18	6.36±0.15	6.53±0.26	6.28±0.22
60 minutes	6.08±0.16	6.41±0.21	6.43±0.17	6.49±0.18	6.77±0.27	6.39±0.22

Table 2 shows the descriptive statistics of plaque pH in all the six chocolate groups. All the groups showed the minimum pH level at 10 minutes. At the end of 60 minutes PWC showed an increase in plaque pH more than the baseline (6.57±0.36 to 6.77±0.27).

Graph 1. Mean distribution of plaque pH

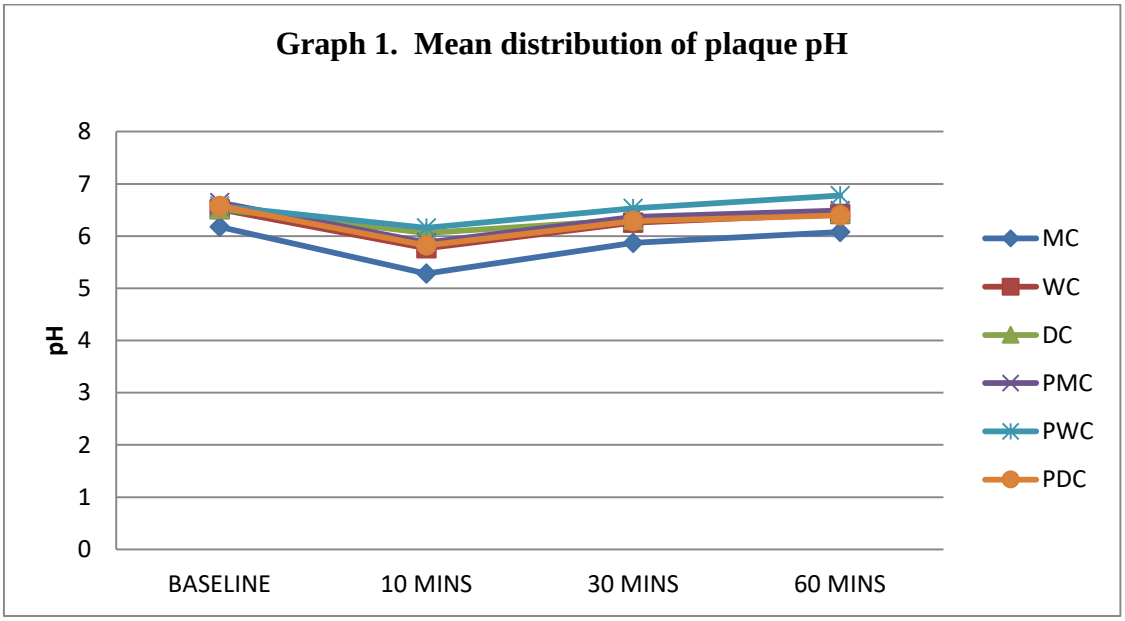


Table 3. Mean distribution of salivary pH in six chocolate groups

Time	MC	WC	DC	PMC	PWC	PDC
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
Baseline	7.32±0.31	7.53±0.27	7.43±0.35	7.65±0.19	7.41±0.21	7.59±0.18
10 minutes	5.45±0.72	6.02±1.05	6.41±0.76	5.65±0.92	7.04±0.40	6.93±0.44
30 minutes	6.61±0.49	7.31±0.35	7.23±0.42	7.14±0.28	7.23±0.33	7.36±0.24
60 minutes	7.23±0.28	7.33±0.24	7.42±0.29	7.36±0.25	7.27±0.23	7.51±0.18

Table 3 shows the descriptive statistics of salivary pH in all the six chocolate groups. All the groups showed the minimum pH level at 10 minutes.

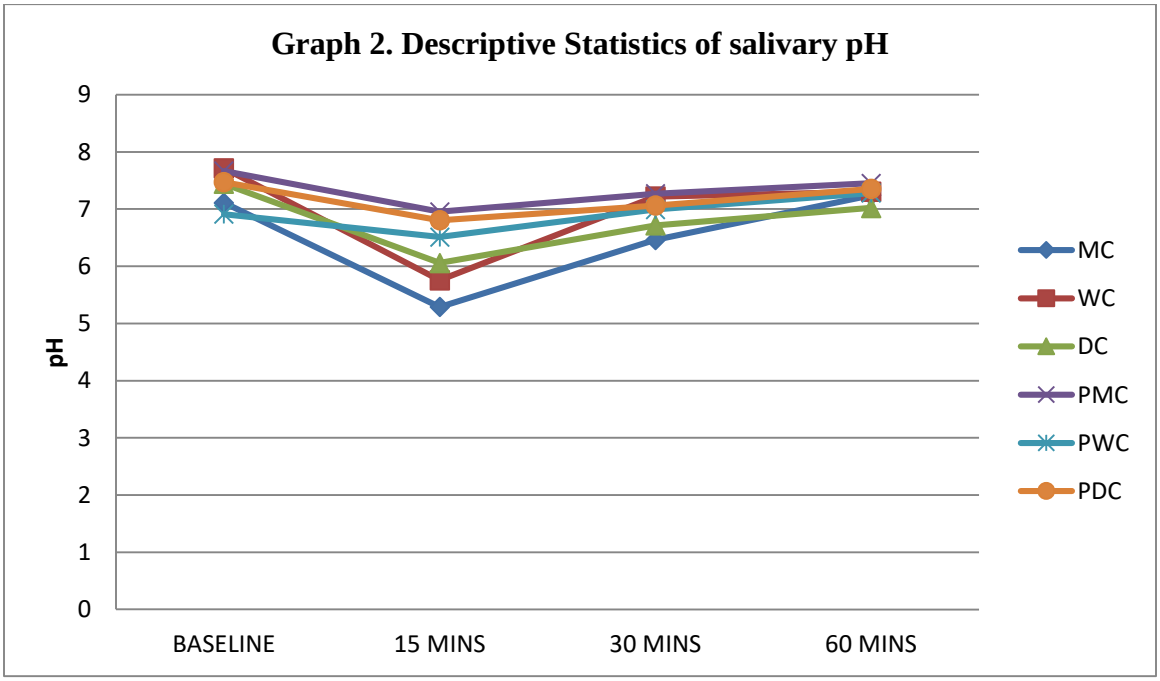


Table 4. Mean distribution for buffering capacity of saliva in six chocolate groups

Time	MC	WC	DC	PMC	PWC	PDC
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
Baseline	7.10±0.35	7.71±0.19	7.43±0.42	7.66±0.14	6.91±1.23	7.46±0.27
10 minutes	5.28±0.75	5.75±1.21	6.05±0.83	6.95±0.22	6.51±0.96	6.80±0.47
30 minutes	6.46±0.52	7.21±0.31	6.71±0.46	7.27±0.25	6.99±0.83	7.06±0.51
60 minutes	7.02±0.28	7.30±0.21	7.01±0.40	7.44±0.20	7.27±0.31	7.35±0.28

Table 4 shows the mean distribution for buffering capacity of saliva in all the six chocolate groups. All the groups had a minimum pH of saliva at 10 minutes. The pH of saliva after buffering action was higher than baseline in PWC.

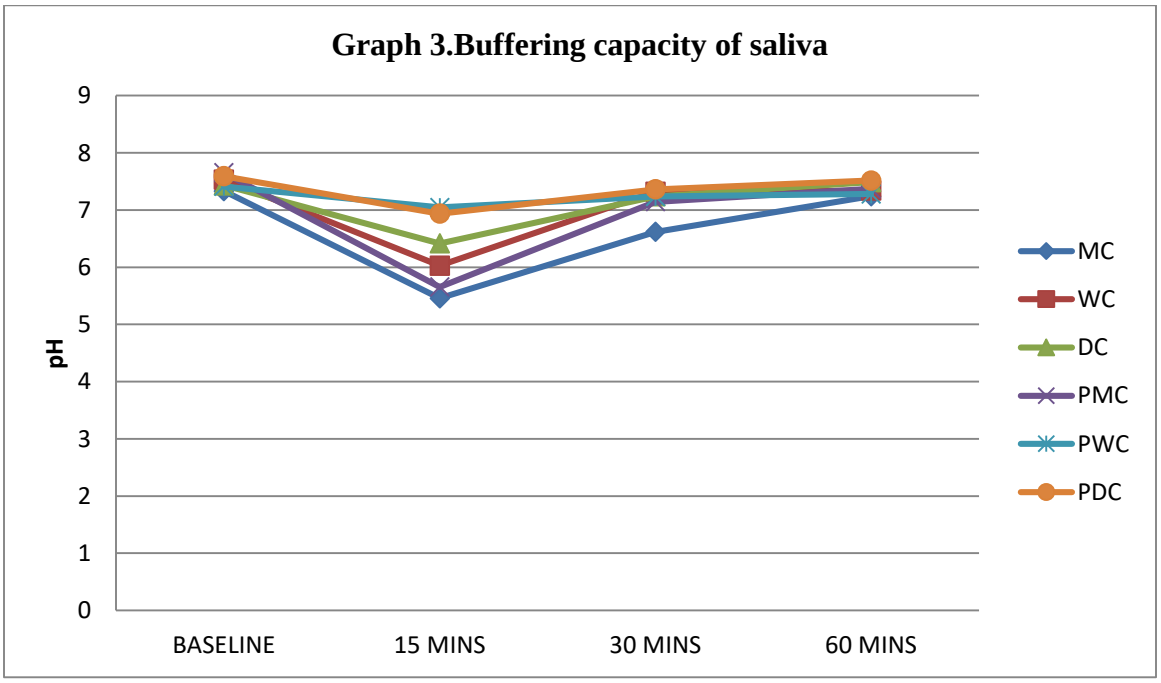


Table 5. Intragroup comparison in the mean difference of plaque pH between different time intervals studied

Groups	Baseline to 10mins		Baseline to 30 mins		Baseline to 60 mins		10 mins to 30 mins		30 mins to 60 mins		10 mins to 60 mins	
	Mean ± SD	p value*	Mean ± SD	p value*	Mean ± SD	p value*	Mean ± SD	p value*	Mean ± SD	p value*	Mean ± SD	p value*
MC	1.87±0.69	<0.001	0.73±0.42	<0.001	0.09±0.18	0.05	-1.16±0.59	<0.001	-0.64±0.47	<0.001	-1.78±0.72	<0.001
WC	1.54±0.83	<0.001	0.33±0.83	<0.001	0.26±0.21	0.001	-1.29±0.77	<0.001	0.00±0.25	<0.001	-1.46±0.83	<0.001
DC	1.25±0.79	<0.001	0.21±0.31	<0.001	-0.07±0.30	0.02	-0.91±0.67	0.002	-0.28±0.23	<0.001	-1.07±0.79	<0.001
PMC	2.00±0.90	<0.001	0.56±0.27	<0.001	0.34±0.22	<0.001	-1.65±0.68	<0.001	-0.31±0.19	<0.001	-1.71±0.81	<0.001
PWC	0.40±0.27	0.002	0.20±0.17	0.635	0.16±0.29	0.11	-0.21±0.30	0.20	-0.04±0.38	0.03	-0.27±0.42	<0.001
PDC	0.66±0.37	<0.001	0.26±0.16	<0.001	0.09±0.15	<0.001	-0.45±0.34	<0.001	-0.17±0.10	<0.001	-0.58±0.36	<0.001

* paired t test

Table 5 shows intragroup comparison in the mean difference of plaque pH between different time intervals studied.

MC, WC, DC, PMC and PDC showed a significant difference, when mean difference of plaque pH was compared between baseline to 10 mins. All the groups except PWC, showed a significant difference in the mean difference of plaque pH between baseline to 30 mins. All the groups except PWC, showed a significant difference in the mean difference of plaque pH between baseline to 30 mins. All the groups except PWC, showed a significant difference in the mean difference of plaque pH between 10 mins to 30 mins. All the groups except PWC, showed a significant difference in the mean difference of plaque pH between 30 mins to 60 mins. All the groups showed a significant difference in the mean difference of plaque pH between 10 mins to 60 mins.

Table 6. Intragroup comparison in the mean difference of plaque pH between different time intervals studied

Groups	Baseline to 10mins		Baseline to 30 mins		Baseline to 60 mins		10 mins to 30 mins		30 mins to 60 mins		10 mins to 60 mins	
	Mean ± SD	p value*	Mean ± SD	p value*	Mean ± SD	p value*	Mean ± SD	p value*	Mean ± SD	p value*	Mean ± SD	p value*
MC	1.87±0.69	<0.001	0.73±0.42	<0.001	0.09±0.18	0.05	-1.16±0.59	<0.001	-0.64±0.47	<0.001	-1.78±0.72	<0.001
WC	1.54±0.83	<0.001	0.33±0.83	<0.001	0.26±0.21	0.001	-1.29±0.77	<0.001	0.00±0.25	<0.001	-1.46±0.83	<0.001
DC	1.25±0.79	<0.001	0.21±0.31	<0.001	-0.07±0.30	0.02	-0.91±0.67	0.002	-0.28±0.23	<0.001	-1.07±0.79	<0.001
PMC	2.00±0.90	<0.001	0.56±0.27	<0.001	0.34±0.22	<0.001	-1.65±0.68	<0.001	-0.31±0.19	<0.001	-1.71±0.81	<0.001
PWC	0.40±0.27	0.002	0.20±0.17	0.635	0.16±0.29	0.11	-0.21±0.30	0.20	-0.04±0.38	0.03	-0.27±0.42	<0.001
PDC	0.66±0.37	<0.001	0.26±0.16	<0.001	0.09±0.15	<0.001	-0.45±0.34	<0.001	-0.17±0.10	<0.001	-0.58±0.36	<0.001

* paired t test

Table 6 shows intragroup comparison in the mean difference of salivary pH between different time intervals studied.

A significant difference was observed in the mean salivary pH of all the chocolate groups at baseline to 10 mins. A significant difference was observed in the mean salivary pH of all the chocolate groups at baseline to 30 mins except PWC. A significant difference was observed in the mean salivary pH in all the chocolate groups at baseline to 60 mins except PWC. A significant difference was observed in the mean salivary pH in all the chocolate groups at 10 mins to 30 mins except PWC. A significant difference was observed in the mean salivary pH in all the chocolate groups at 30 mins to 60 mins except PWC. A significant difference was observed in the mean salivary pH of all the chocolate groups at 10 mins to 60 mins.

Table 7. Intragroup comparison in the mean difference of buffering capacity of saliva between different time intervals studied

Groups	Baseline to 10mins		Baseline to 30 mins		Baseline to 60 mins		10 mins to 30 mins		30 mins to 60 mins		10 mins to 60 mins	
	Mean ± SD	p value*	Mean ± SD	p value*	Mean ± SD	p value*	Mean ± SD	p value*	Mean ± SD	p value*	Mean ± SD	p value*
MC	1.82±0.71	<0.001	0.64±0.48	<0.001	-0.13±0.19	0.01	1.17±0.58	<0.001	0.77±0.50	<0.001	-1.95±0.72	<0.001
WC	1.95±1.16	<0.001	0.49±0.27	<0.001	0.41±0.18	<0.001	-1.46±1.10	<0.001	-0.08±0.13	<0.001	-1.54±1.16	0.561
DC	1.38±0.80	<0.001	0.72±0.34	<0.001	0.42±0.38	0.183	-0.652±0.83	<0.001	-0.30±0.15	<0.001	-0.96±0.88	<0.001
PMC	0.71±0.26	<0.001	0.39±0.27	<0.001	0.21±0.24	<0.001	-0.31±0.21	<0.001	-0.17±0.14	<0.001	-0.49±0.22	<0.001
PWC	0.40±0.89	<0.001	-0.08±0.93	0.635	-0.36±1.23	0.01	-0.48±1.06	0.001	-0.28±0.72	0.505	-0.76±0.91	0.005
PDC	0.66±0.32	<0.001	0.40±0.33	<0.001	0.11±0.14	0.007	-0.25±0.27	<0.001	-0.29±0.33	<0.001	-0.54±0.31	<0.001

* paired t test

Table 7 shows intragroup comparison in the mean difference of buffering capacity of saliva between different time intervals studied.

All the chocolate groups showed a significant difference in the mean difference of buffering capacity of saliva between baseline to 10 mins. All the groups except DC, showed a significant difference in the mean difference of buffering capacity of saliva between baseline to 30 mins. All the groups except DC, showed a significant difference in the mean difference of buffering capacity of saliva between baseline to 60 mins. All the groups showed a significant difference in the mean difference of buffering capacity of saliva between 10 mins to 30 mins. All the groups except PWC, showed a significant difference in the mean difference of buffering capacity of

saliva between 30 mins to 60 mins. All the groups showed a significant difference in the mean difference of buffering capacity of saliva between 10 mins to 60 mins.

Table 8. Comparison of plaque pH between the six chocolate groups at all the time intervals studied

Time	p value*
Baseline to 10mins	**<0.001**
Baseline to 30 mins	**<0.001**
Baseline to 60 mins	**<0.001**
10 mins to 30 mins	**0.026**
30 mins to 60 mins	**<0.001**
10 mins to 60 mins	**0.005**

*ANOVA

Table 8 shows the intergroup comparison of plaque pH between the six chocolate groups in different time periods. Statistically significant differences in plaque pH were observed among the groups at all the time intervals studied.

Table 9. Intergroup comparison for plaque pH among the six chocolate groups at different time intervals

Group	Group	Baseline to10 mins p value*	Baseline to 30 mins p value*	Baseline to 60 mins p value*	10 mins to 30 mins p value*	30 mins to 60 mins p value*	10 mins to 60 mins p value*
MC	WC	1.000	1.000	1.000	1.000	1.000	1.000
	DC	**0.008**	1.000	1.000	0.017	0.160	0.160
	PMC	1.000	1.000	1.000	1.000	0.192	0.192
	PWC	**0.002**	**<0.001**	**<0.001**	0.589	1.000	1.000
	PDC	1.000	1.000	1.000	1.000	0.057	0.057
WC	DC	0.296	1.000	1.000	0.294	1.000	1.000
	PMC	1.000	1.000	1.000	1.000	1.000	1.000
	PWC	0.105	**0.004**	**<0.001**	1.000	0.371	0.371
	PDC	1.000	1.000	1.000	1.000	1.000	1.000
DC	PMC	0.201	1.000	1.000	0.272	1.000	1.000
	PWC	1.000	0.066	**<0.001**	1.000	.013	0.013
	PDC	0.268	1.000	1.000	0.506	1.000	1.000
PMC	PWC	0.068	**0.002**	**<0.001**	1.000	0.016	0.016
	PDC	1.000	1.000	1.000	1.000	1.000	1.000
PWC	PDC	0.094	**0.001**	**<0.001**	1.000	**0.004**	**0.004**

*Bonferroni post hoc test

Table 9 shows intergroup comparison in plaque pH at all the time intervals. From baseline to 10 mins there was significant difference between groups MC and DC, MC and PWC. From baseline to 30 minutes there was a significant difference between groups MC and PWC, WC and PWC, PMCand PWC and PWC and PDC. From baseline to 60 mins there was a significant difference between groups MC and PWC, WC and PWC and DC and PWC, PMC and PWC and PWC and PDC. From 10 mins to 30 mins there was no significant difference between the groups. From 30 mins to 60 mins there was a statistically significant difference between groups PWC and PDC. From 10 mins to 60 mins there was a statistically significant difference between groups PWC and PDC.

Table 10. Comparison of salivary pH between the six chocolate groups at different time intervals studied

Time	p value*
Baseline to 10 mins	**<0.001**
Baseline to 30 mins	**0.003**
Baseline to 60mins	**<0.001**
10 mins to 30 mins	**<0.001**
30 mins to 60 mins	0.132
10 mins to 60 mins	**<0.001**

*ANOVA

Table 10 shows the intergroup comparison of salivary pH at all the time intervals studied. A statistically significant difference was observed in all the time intervals studied, except 30 mins to 60 mins.

Table 11. Intergroup comparison of salivary pH among the six chocolate groups at different time intervals

Group	Group	Baseline to 10 mins p value*	Baseline to 10 mins p value*	Baseline to 60 mins p value*	10 mins to 30 mins p value*	30 mins to 60 mins p value*	10 mins to 60 mins p value*
MC	WC	1.000	1.000	0.930	1.000	0.173	1.000
	DC	1.000	**.006**	0.260	1.000	1.000	**0.001**
	PMC	1.000	1.000	**0.007**	0.034	1.000	1.000
	PWC	0.020	0.178	1.000	**<0.001**	1.000	**<0.001**
	PDC	**0.007**	1.000	1.000	**<0.001**	1.000	**<0.001**
WC	DC	0.141	0.229	**0.001**	0.258	1.000	**0.008**
	PMC	1.000	1.000	1.000	0.357	1.000	1.000
	PWC	0.450	1.000	1.000	**<0.001**	0.518	**<0.001**
	PDC	0.199	1.000	1.000	**<0.001**	1.000	**<0.001**
DC	PMC	1.000	0.023	**<0.001**	**<0.001**	1.000	**.003**
	PWC	**<0.001**	1.000	0.038	0.001	1.000	**<0.001**
	PDC	**<0.001**	0.663	0.405	0.072	1.000	0.061
PMC	PWC	**0.006**	0.499	0.020	**<0.001**	1.000	**<0.001**
	PDC	**0.002**	1.000	0.021	**<0.001**	1.000	**<0.001**
PWC	PDC	1.000	1.000	1.000	1.000	1.000	1.000

*Bonferroni post hoc test

Table 11 shows intergroup comparison in salivary pH among the six chocolate groups at all the time intervals. From baseline to 10 mins there was significant difference between group MC and PDC, DC and PWC, DC and PDC, PMC and PWC, PWC and PDC. From baseline to 30 minutes there was a significant difference between groups MC and DC. From baseline to 60 mins there was a significant difference between groups MC and PMC, WC and DC and DC and PMC. From 10 mins to 30 mins there was a significant difference between groups MC and PWC, MC and PDC, WC and PWC, WC and PDC, DC and PMC, PMC and PWC, PMC and PDC. From 30 mins to 60 mins there was no statistically significant difference. From 10 mins to 60 mins there was a statistically significant difference between groups MC and DC, MC and PWC,

MC and PDC, WC and DC,WC and PDC, DC and PMC, DC and PDC, PMC and PWC, PMC and PDC.

Table 12. Comparison of buffering capacity of saliva between the six chocolate groups at different time intervals studied

Time	p value*
Baseline to 10 mins	**<0.001**
Baseline to 30 mins	**<0.001**
Baseline to 60mins	**<0.001**
10 mins to 30 mins	**<0.001**
30 mins to 60 mins	**<0.001**
10 mins to 60 mins	**<0.001**

*ANOVA

Table 12 shows the intergroup comparison of buffering capacity of saliva at all the time intervals studied. A statistically significant difference in buffering capacity of saliva was seen between all the groups in all the time intervals studied.

Table 13. Intergroup comparison of buffering capacity of saliva among the six chocolate groups at different time intervals

Group	Group	Baseline to 10 mins P value*	Baseline to 30 mins P value*	Baseline to 60 mins P value*	10 mins to 30 mins P value*	30 mins to 60 mins P value*	10 mins to 60 mins P value*
MC	WC	1.000	1.000	**0.003**	**<0.001**	1.000	0.672
	DC	0.426	1.000	**0.002**	**<0.001**	0.160	**<0.001**
	PMC	**<0.001**	0.809	0.235	**<0.001**	0.192	**<0.001**
	PWC	**<0.001**	**<0.001**	1.000	**<0.001**	1.000	**<0.001**
	PDC	**<0.001**	0.967	1.000	**<0.001**	0.057	**<0.001**
WC	DC	0.062	1.000	1.000	0.503	1.000	0.061
	PMC	**<0.001**	1.000	1.000	1.000	1.000	**<0.001**
	PWC	**<0.001**	**<0.001**	**<0.001**	0.758	0.371	**.002**
	PDC	**<0.001**	1.000	0.587	0.699	1.000	**<0.001**
DC	PMC	0.013	0.167	1.000	1.000	1.000	0.333
	PWC	**<0.001**	**<0.001**	**<0.001**	1.000	0.013	1.000
	PDC	**0.005**	0.208	0.485	1.000	1.000	.625
PMC	PWC	1.000	**0.004**	**0.001**	1.000	0.016	1.000
	PDC	1.000	1.000	1.000	1.000	1.000	1.000
PWC	PDC	1.000	**0.003**	0.012	1.000	**0.004**	1.000

* Bonferroni post hoc0

Table 13 shows intergroup comparison of buffering capacity between the groups at different time intervals. From baseline to 10 mins groups MC and PMC, MC and PWC, MC and PDC, WC and PMC, WC and PWC, WC and PDC, DC and PWC, DC and PDC showed a significant difference. From baseline to 30 mins MC and PWC, WC and PWC, DC and PWC, PMC and PWC and PWC and PDC showed a significant difference. From baseline to 60 mins MC and WC, MC and DC, WC and PWC, DC and PWC, PMC and PWC was significant. From 10 to 30 mins MC and WC, MC and DC, MC and PMC, MC and PWC, MC and PDC showed a significant. From 30 to 60 mins there was significant difference between PWC and PDC. From

10 to 60 mins MC and DC, MC and PMC, MC and PWC, MC and PDC, WC and PMC, WC and PWC along with WC and PDC showed a significant difference.

Table 14. Intragroup comparison of *S.mutans* colony count for Group I at different time intervals

Time	Mean difference 10^3 CFU	p value*	Percentage reduction
Baseline to post intervention	71.70	**<0.001**	25.40
Baseline to 15 days	58.35	**<0.001**	20.85
Baseline to 30 days	31.95	**<0.001**	11.50
Post intervention to 15 days	-13.35	0.28	-9.53
15 days to 30 days	-39.75	**<0.001**	-24.70
Post intervention to 30 days	-26.40	**<0.001**	-11.28

* Wilcoxon signed rank test

Table 16 shows the intragroup comparison of *S.mutans* colony count in Group I at various time intervals. A statistically significant difference was found at baseline to post intervention ($p<0.001$), baseline to 15 days ($p<0.001$) and baseline to 30 days($p<0.001$). No significant difference was found from post intervention to 15 days. From 15 days to 30 days and post intervention to 30 days there was a negative mean difference and percentage reduction in colony count which was statistically significant, indicating increase in colony count. The highest reduction in colony count was found between baseline to post intervention (25.4%) followed by baseline to 15 days (20.8%).

Table 15. Intragroup comparison of *S.mutans* colony count for Group II at different intervals

Time	Mean difference 10^3 CFU	p value*	Percentage reduction
Baseline to post intervention	29.93	**<0.001**	106.75
Baseline to 15 days	25.48	**0.001**	95.40
Baseline to 30 days	19.10	**0.001**	69.15
Post intervention to 15 days	-8.53	0.48	-11.35
15 days to 30 days	-21.66	**0.001**	-37.60
Post intervention to 30 days	-8.78	**0.002**	-26.25

* Wilcoxon signed rank test

Table 16 shows the intragroup comparison of *S.mutans* colony count in Group II at various time intervals. A statistically significant difference was found at baseline to post intervention ($p<0.001$), baseline to 15 days ($p<0.001$) and baseline to 30 days ($p<0.001$). No significant difference was found from post intervention to 15 days. From 15 days to 30 days and post intervention to 30 days there was a negative mean difference and percentage reduction in colony count which was statistically significant, indicating increase in colony count. The highest reduction in colony count was found between baseline to post intervention (106.7%) followed by baseline to 15 days (95.4%).

Table 16. Intragroup comparison of *S.mutans* colony count for Group III at different time intervals

Time	Mean difference Xx 10^3 CFU	p value*	Percentage reduction
Baseline to post intervention	53.11	**<0.001**	152.35
Baseline to 15 days	43.56	**<0.001**	133.50
Baseline to 30 days	31.35	**<0.001**	103.30
Post intervention to 15 days	-149.96	**0.003**	-18.85
15 days to 30 days	-279.72	**<0.001**	-30.20
Post intervention to 30 days	-18.06	**<0.001**	-49.05

* Wilcoxon signed rank test

Table 16 shows the intragroup comparison of *S.mutans* colony count in group III at various time intervals. A statistically significant difference was found at baseline to post intervention ($p<0.001$), baseline to 15 days($p<0.001$) and baseline to 30 days($p<0.001$). No significant difference was found at post intervention to 15 days. At 15 days to 30 days and post intervention to 30 days there was a negative mean difference and percentage reduction in colony count which was statistically significant, indicating increase in colony count. The highest reduction in colony count was found between baseline to post intervention (152.3%) followed by baseline to 15 days (133.5%).

Table 17. Intergroup comparison of *S.mutans* colony count between groups at all the time intervals studied

Group	Group	Baseline to post intervention p value*	Baseline to 15 days p value*	Baseline to 30 days p value*	Post intervention to 15 days p value*	15 days to 30 days p value*	Post intervention to 30 days p value*
1	2	0.694	0.558	0.265	1.000	1.000	1.000
	3	0.022	0.26	**0.005**	1.000	1.000	1.000
2	3	0.363	0.522	0.351	1.000	1.000	1.000

*Bonferroni post hoc test

Table 17 shows intergroup comparison of *S.mutans* colony count between the groups at all the time intervals studied**.** There was no statistically significant difference between the groups, expect probiotic milk chocolate and probiotic dark chocolate from baseline to 30 days.

Figure 1. Milk chocolate group showing antimicrobial activity against *S.mutans*

Figure 2. White chocolate group showing antimicrobial activity against *S.mutans*

Figure 3. Dark chocolate group showing antimicrobial activity against *S.mutans*

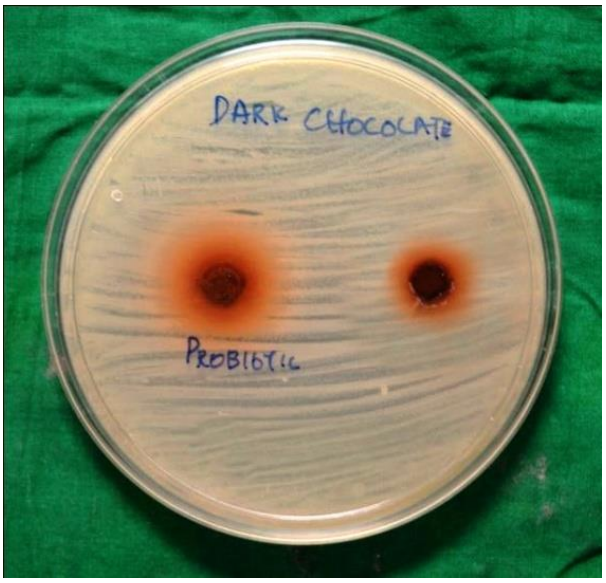


Figure 4. TSBB agar showing growth of *S.mutans*

DISCUSSION

Rugg-Gunn et al[75] conducted a study in England and reported that each child consumed a mean of 35 g of chocolate per day. Children's affinity to chocolates is very strong, and trying to break the bond often results in failure. The age group of 8-12 years was selected because the consumption of chocolates among school going children was observed to be higher than in adolescence.[58] Incorporation of probiotics into chocolate could be beneficial to children of this age group who are known to be fond of chocolates. These children belong to the concrete operational stage of Jean Piaget's cognitive theory. They abide the rules, which make them follow the instructions given to them.[68]

Selection of chocolates

Chocolates chosen for the study were the representatives of milk, white and dark chocolate variants. Verakaki and Duggal[94] compared various chocolates in the European market and found milk chocolate with hazelnut and plain milk chocolate had low acidogenic potential. Nirmala et al compared various filled and unfilled chocolates and found that filled chocolates were more acidogenic. Unfilled plain chocolates were chosen for the present study to avoid effects of other ingredients which could alter the original action of the chocolate. Chocolates used in the present study contained various ingredients. Milk chocolate contained cocoa mass, cocoa butter, milk, vegetable fat, emulsifiers and sugar. Dark chocolate contained 50% cocoa solids, cocoa butter and sugar and white chocolate contained milk solids, hydrogenated vegetable fat, soya lecithin and sugar.

Selection of probiotic

A healthy oral cavity is colonized by a large number of diverse bacteria. The relationship between the oral microbiota and the host is generally harmonious and symbiotic. Changes in the oral environment bring about deleterious shifts in this microbiome, leading to dysbiosis or

bacterial imbalance.[47] The use of replacement therapies would help to maintain the equilibrium of the biofilm and thereby foster health.[91] The commonly studied probiotic in the oral cavity are *L.reuteri*,[57,13] *L. rhamnosus*,[55,2,83,72] and *L. acidophilus.*[78,85]

The probiotic used for the present study was *L.sporogenes* which is a spore forming bacteria. Spores are more resistant to adverse environmental conditions thus allowing it to undergo industrial processing.[76] This enables the viability even in extreme temperature ranges, reduced cost of production and extended shelf life.[42] The probiotic used in this study was the commercially available Bifilac^{TM} sachet. It contains probiotic along with prebiotic bacteria which act synergistically in symbiosis. Literature search showed its proven effect in minimal hepatic encephalopathy,[92] acute viral diarrhea[54,4] efficacy against *porphyromonas gingivalis*[23] and *S.mutans.*[21]

The preparation of chocolate was done in accordance to the FDA guidelines which recommends at least 10^8 CFU of bacteria to attain its benefits. Kanafari et al [39] and posseimers et al[69] used similar concentrations of probiotic for chocolate preparation. Kanafari et al[39] in an invitro trial showed the inhibitory potential of *L.plantarum* containing probiotic dark chocolate on *S. mutans* colony count. It was in accordance with the results of the present invitro trial which showed that all types of chocolates added with probiotics had inhibitory effect on *S.mutans*. The maximum zone of inhibition was obtained for probiotic dark chocolate. There was no zone of inhibition for plain milk chocolate. However literature search did not reveal any studies which dealt with acidogenic and cariogenic properties of probiotic chocolates. Hence this trial was planned to include 2 parts. Part 1evaluated the effectiveness of chocolates on plaque ph, salivary pH and buffering capacity of saliva. Part 2 of the trial evaluated its effectiveness against *S.mutans* by administering chocolates for 5 consecutive days.

Culture media

The most commonly used selective media for *S.mutans* is Mitis Salivarius Bacitracin agar, which was modified by Kimmel and Tinanoff[40] as Mitis salivarius agar with kanamycin sulfate, sorbitol, potasium tellurite and bacitracin (MSKB). Llena et al[45] added 5% potassium tellurite and 64 µg/ml fluconazol to MSKB. Many other medias such as Trypticase soy with sucrose and bacitracin (TSY 20B), *Staat*[82] and glucose-sucrose-tellurite-bacitracin (GSTB) *Tanzer*[88] are available. The culture media used for this study was Thioglycolate Sucrose Blood Bacitracin agar (TSBB). Singh et al[79] formulated the selective medium TSBB and compared it with the other selective medias. Results indicated that TSBB medium inhibits the all *Streptococcus* species other than *S.mutans* present in the oral cavity. Sodium thioglycolate present in the medium consumes oxygen, making it an anaerobic medium which does not require special anaerobic incubator for the growth of *S.mutans*.

Plain chocolates

Considering the plaque pH from baseline to 10 mins, 30 mins and 60 mins , DC showed the least fall in pH. The acidogenicity of DC was the least followed by WC and MC. Immediate fall in pH after 10 mins of consumption of chocolates was high in MC and this was significantly different from DC. On observing the salivary pH from 10 mins, 30 mins and 60 mins, DC showed the least fall in pH. The acidogenicity of DC was the least followed by WC and MC. The fall in pH after 30 mins of consumption of chocolates was high in MC and was significantly different from DC. The fall in pH after 60 mins of consumption of chocolates was low in DC and was significantly different from MC. On comparing the buffering capacity of saliva from baseline to 10 mins, DC had the least fall in pH followed by MC and WC. From baseline to 30 mins, WC had the least fall followed by MC and DC. From baseline to 60 mins,

MC had the maximum raise in salivary pH which was significantly different from WC and MC. Results favor DC to be less acidogenic followed by WC and MC.

The probable reason for less acidogenicity of dark chocolates is that it contains flavanols. They are a subclass of flavonoids which are, in turn, a subclass of polyphenols. Flavanols appear as monomers and those found in cocoa beans are epicatechin and catechin. They can exist as dimmers or polymers, commonly referred as polyphenols. In a study by Percival et al[66] cocoa polyphenols were tested against the growth of *S.mutans*. It was found that acid production of *S.mutans* from sucrose was significantly inhibited and the rate of pH change was markedly reduced. Further study by Oswaka et al[63] found two types of cariostatic substances in dark chocolate, higher molecular- weight polyphenolic compounds which showed anti-Glucosyltransferase activities and unsaturated free fatty acids, such as linoleic and oleic acids which had antibacterial activity against *S. mutans.*

Our results were in accordance with Frostell[27] who carried out experiment with chocolates containing different concentrations of sucrose and cocoa. They showed that chocolates with high cocoa content and low sugar had a increase in plaque pH. Amith et al also found that dark chocolate to have the least pH drop on comparison with milk, white and caramel chocolate. Edgar et al[22] and Rugg-Gunn et al[74] showed both milk and dark chocolates to be less cariogenic compared with other food stuffs. However Grenby and Mistry[30] and Nirmala et al[58] identified dark chocolate to be more cariogenic than milk chocolate, which was contradictory to our results.

Probiotic chocolates

Considering the plaque pH from baseline to 10 mins, 30 mins and 60 mins, PWC showed the least fall in pH. The acidogenicity of PWC was the least followed by PDC and PMC. From

baseline to 30 mins, the least fall in pH was found for PWC which was significantly different from PDC and PMC. In terms of salivary pH from baseline to 10 mins and 30 mins, PWC showed the least fall in pH. From baseline to 30 mins, the maximum rise in pH was found for PWC which was significantly different from PDC and PMC. The acidogenicity of PWC was the least followed by PDC and PMC. The buffering capacity of saliva was higher for PWC from baseline to 10 mins, 30 mins and 60 mins. The acidogenecity of PWC was least followed by PDC and PMC.

The higher pH raise in PWC could be attributable to its milk content. Milk components such as casein phosphopeptides (CPP) and glycomacropeptide (GMP) get incorporated into the salivary pellicle and reduce the adherence of *S. sobrinus* and *S. mutan.* By selectively inhibiting adhesion, these dairy bioactives could modulate the microbial composition of dental plaque. This could in turn control acid formation in dental plaque, thus reducing hydroxyapatite dissolution from tooth enamel.[56] Jenkins and Ferguson[35] conducted invitro comparisons of 4 % lactose solutions with cow's milk. They found a negligible fall in plaque pH which was due to milk's high buffering capacity. High levels of calcium and phosphate could have contributed to the lesser dissolution of enamel.

In the present study addition of probiotics to white chocolate was less acidogenic than dark chocolate. Composition of chocolates plays a major role in the retention of chocolates on the tooth surface and their oral clearance. Dark chocolates require relatively greater chewing phase and oral processing time whereas milk chocolate have a lower solid fat index. Milk chocolate is ready to swallow than dark chocolate and requires lesser chewing time for a swallow able state.[28] So WC which contains more of milk, will be swallowed readily and hence contribute less to decrease in pH drop.

Plain chocolate vs probiotic chocolate

Estimation of acidogenecity of probiotics were studied by Sudhir et al[85] and Srivatsav et al[81] following consumption of probiotic curd. The former found a decrease in salivary pH, whereas the later found an increase in salivary pH. In the present study, irrespective of the type of chocolate used, probiotic chocolates were better in salivary, plaque pH and buffering capacity of saliva than their normal counterparts.

DC Vs PDC

In terms of plaque pH, salivary pH and buffering capacity of saliva, PDC showed the least fall in plaque pH compared to DC at all the time intervals studied. Salivary pH and buffering capacity of saliva from baseline to 10 mins showed significant increase in pH for PDC than DC.

MC Vs PMC

PMC showed least fall in plaque pH, salivary pH and buffering capacity of saliva at all the time intervals studied. Salivary pH from baseline to 60 mins showed a significant difference between PMC and MC. Buffering capacity of saliva from baseline to 10 mins showed a significant increase in buffering for PMC compared to MC.

WC Vs PWC

PWC showed least fall in plaque pH, salivary pH and buffering capacity of saliva at all the time intervals studied. PWC showed a significant increase in plaque pH from baseline to 30 mins. Buffering capacity of saliva showed a significant increase in pH from baseline to 10mins, 30 mins as well as 60 mins.

One of the proposed mechanisms of action of probiotics is its production of lactic acid, acetic acid, hydrogen peroxide and bacteriocins which in turn reduce the plaque pH. In the

present study, the probioitic used reduced the pH drop in plaque and salivary pH which were either similar or lesser than the normal chocolates. Production of lactic acid by bacteria depends on the type of sugar and bacterial strain.[38] Hedgber[33] showed that L. paracasei and L. reuteri did not ferment any kind of sugar. L. rhamnosus exhibited negative reaction for sucrose and L. plantarum fermented sucrose. Literature search did not reveal any proof for the production of acids by the probiotic bacteria used in the present study. Accordingly the results of this study also indicated that there was no significant acid production by probiotic bacteria, as the ph drop in plaque and saliva were minimal in probiotic chocolate groups compared to their counterparts.

Effect on S.*mutans* colony count

The effect of probiotic against *S.mutans* was studied by different vehicles. Nase et al[55] was the first to conduct a long term randomized controlled trial using probiotic milk. The children in their experimental group showed a reduction in the *S.mutans* counts after 7 months of intervention.

The present study was of a short duration with probiotic intake for 5 consecutive days. Similar studies with short term administration of probiotics for 7 days have been carried out by various researchers and proved the efficacy of probiotics,[20,6,81,3,46] Chinnappa et al[16] assessed the *S.mutans* colony count with probiotic ice cream and probiotic yoghurt after 7 days administration. They found a significant reduction in the *S.mutans* colony count at 1 hour and 7 days post intervention in both the probiotic groups.

Intra group comparison of bacterial colony count showed a significant reduction in the number of colonies after 30 days. All the probiotic chocolate groups showed maximum decrease in bacterial colony count at the post intervention period. However from post intervention to 15

days the colony count showed a steady increase but did not reach the baseline. Results indicated the short term action and easy wash out of probiotics on cessation of its intake.

The results were in accordance with Ghasehmi et al[28] who investigated the effect of probiotic yoghurt and xylitol chewing gum on salivary *S.mutans* colony count after 3 weeks intervention. The children were followed up for 4 weeks. Results showed a reduction in colony count upto 2 weeks, followed by an increase in colony count in the 4th week which was however lesser than the baseline. Chuang et al[17] showed similar results after administering probiotic tablets for 2 weeks. Ashwin et al[3] conducted a similar study by administering probiotic ice cream for 7 consecutive days and were followed up for a period of 6 months. There was a decrease in the colony count till 30 days. Results of the present study were in contrast to Mahantesha et al[46] who conducted a randomized controlled trail by administering probiotic milk and ice cream for 7 consecutive days. Their results showed a reduction in colony count in both the groups at 1 month, however the probiotic ice cream group showed a reduced colony count even after 3 months.

The probable mechanism of action of probiotics include; competitive inhibition, production of bacteriocins, organic acids, hydrogen peroxide, modulating pH and modulating redox potential.[7] In this study the probiotics might have competitively inhibited *S.mutans*.[77] However from the results of the present study and previous literature, it is questionable or not they have any residual effect after discontinuation of intake. Systematic review by Cagetti et al[12] evaluated the effect of probiotics administration on caries risk factors and showed that in two-thirds of the selected papers, probiotics have demonstrated the capacity to reduce Mutans sterptococci counts in saliva and/or plaque in short-term. Probiotic bacteria can only transiently colonize the oral cavity during the use of probiotic products. [52,41,96] The retention of probiotic is

an important factor to be taken into account. Retention observed depends on the products, strains and also host individuals. [11,67,41,96] Probiotics are given even to newborns because of their beneficial health effects but information on their oral colonisation in children is not available.[70,87]

Results of the present study were contradictory to the findings of Lexner et al[44] who compared normal milk and milk supplemented with bacterium L. rhamnosus LB21 for 2 weeks. They did not observe any significant difference in the salivary colony counts.

At baseline to 30 days probiotic dark chocolate (103%) showed a significant reduction in colony count than probiotic dairy milk (11%). The mechanism for the observed inhibitory effect of the cocoa polyphenols on biofilm formation and acid production is not clear. It has been proposed that polyphenols could inhibit enzymes necessary for the production of energy in cells or may cause changes in cell membrane permeability.[1] Evidence has shown that cocoa extracts can act against mutans streptococci by inhibiting sucrose-dependent adherence, glucosyltransferase activity and insoluble glucan synthesis.[62,64,63] Cocoa butter is one of the basic ingredients of a cocoa bean. It consists mainly of a triglyceride ester form of palmitic, stearic, oleic, and linoleic acids which were found to be antimicrobial agents.[63]

Probiotics have shown beneficial health impact in humans, leading to several new recommendations for its use. Chocolates can serve as a vehicle for delivering probiotics in children making it beneficial for health. Probiotic chocolates have the added advantage of reducing the chocolates acidogenicity and hence making them tooth friendly.

Limitations

- Evaluating the adherence of the probiotic bacteria in dental plaque and rate of its acid production has not been studied. This could have given a clear insight on its mechanism of action.

- Larger sample size of children with different caries status could have been assessed, to get a better picture of the anticariogenic properties of probiotic chocolates.
- Accepted markers for caries such as caries increment or incidence can been assessed with long term studies.

SUMMARY AND CONCLUSION

The present randomized controlled trial was conducted in the Department of Pediatric and Preventive Dentistry, K.S.R Institute of Dental Science and Research (KSRIDSR). The study was planned and organized in association with various schools in Tiruchengode to determine the plaque pH, salivary pH, buffering capacity and *S. mutans* count after consumption of custom made probiotic chocolates in children. Ninety children with DMFT/dmft ≤ 3 were included for the study. In phase 1 children were randomly divided into 3 equal groups (n=30): (i) Milk chocolate (MC), (ii) White chocolate (WC), (iii) Dark chocolate (DC). Phase 2 of the trial was done after a wash out period of 20 days. The children assigned to the milk, white and dark chocolates were assigned to their respective probiotic groups. Plaque pH, salivary pH and buffering capacity of saliva were measured at baseline, 10 mins, 30 mins and 60 mins post consumption of chocolates using pH meter after one time consumption of chocolates. For the second part of the study 60 children with same inclusion criteria were randomly divided into 3 equal groups (n=20) Group 1- Probiotic dairy millk, Group 2- Probiotic milky bar and Group 3- Probiotic dark chocolate. Children were provided with respective chocolates for 5 consecutive days in a week. Salivary samples were collected at baseline, post intervention, 15 days and 30 days post intervention. Saliva cultures were done in TSBB media and colony count was done after 48- 72 hours of incubation period. The results were tabulated and analyzed statistically

The following findings can be inferred from the study:

- Among the plain chocolates, DC showed the least acidogenecity followed by WC and MC.
- Among the probiotic chocolates, PWC showed the least acidogenecity followed by PDC and PMC.
- PMC was found to be less acidogenic than MC.

- PWC was found to be less acidogenic than WC.
- PDC was found to be less acidogenic than MC.
- *S.mutans* colony count reduced in all the three probiotic chocolate groups.
- Maximum reduction in colony count was observed from baseline to immediate post intervention period in all the three probiotic chocolate groups.
- The colony count increased after 15days post intervention but did not raise above baseline colony count in all the three chocolate groups.
- PDC showed the maximum reduction in the *S.mutans* colony count among the probiotic chocolates.
- Invitro evaluation of chocolates against *S.mutans* showed inhibitory zones for all the probiotic chocolates. The maximum zone of inhibition was observed for probiotic dark chocolate. Plain dark chocolate and white chocolate also showed inhibitory effect against *S.mutans.*

CONCLUSION

- PWC was found to be least acidogenic among all the six chocolate groups studied.
- DC was found to be least acidogenic among normal chocolates.
- Probiotic chocolates were less acidogenic than their counterparts
- All probiotic chocolates were effective in reducing the *S.mutans* colony count.
- Probiotic dark chocolate showed the highest percentage reduction in *S.mutans* colony count.

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APPENDIX

APPENDIX – I

INSTITUTIONAL ETHICAL COMMITTEE

KSR INSTITUTE OF DENTAL SCIENCE & RESEARCH

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Chairman
Dr. P. PONMURUGAN, Ph.D.,
Prof. & Head Dept. of Biotechnology
KSR College of Technology,
KSR Kalvi Nagar, Tiruchengode.

Member Secretary
Dr. G.S. KUMAR, MDS.,
Principal,
KSR Institute of Dental Science & Research,
KSR Kalvi Nagar, Tiruchengode.

Members

Dr.G.Ayppadasan, Ph.D.,
Biotechnologist

Mr.A.Thirumoorthi, M.A.B.L.,
Human Activist

Dr.R.Renuka, M.D.S.,(Perio),M.Sc.,
Family Counsellor

Dr.K.Sivakumar, MDS.,(Cons.Dent.)

Dr.Suman, M.D.S.,(OMDR)

Dr.Sharath Ashokan, MDS.,(Pedo)

Dr.G.Rajeswari, Ph.D.,(Biochemistry)

Dr.K.Karthick, MDS.,(Cons.Dent.)

Mr.V.Mohan, M.Sc.,M.Phil.,(Physicist)

Mr.A.P.S.Raja, B.A.,
(Layperson)

Ref.: 144/KSRIDSR/EC/2015 **Date : 19.09.2016**

To

Dr.R.G.Janani,
Postgraduate Student,
Dept. of Peadodontics,
KSR Institute of Dental Science & Research,

*****

Your dissertational study titled "Effect of custom made probiotic chocolates on Streptococcus mutans, plaque pH salivary pH and buffering capacity in children – A Randomized controlled trial" presented before the ethical committee on 15th Sep. 2016 has been discussed by the committee members and has been approved.

You are requested to adhere to the ICMR guidelines on Biomedical Research and follow good clinical practice. You are requested to inform the progress of work from time to time and submit a final report on the completion of study.

Signature of Member Secretary
(Dr.G.S.Kumar)

APPENDIX – II

URKUND

Urkund Analysis Result

Analysed Document:	PLAGARISM JANANI RG.docx (D34390804)
Submitted:	1/5/2018 7:20:00 PM
Submitted By:	jaanu.krishnan.16@gmail.com
Significance:	1 %

Sources included in the report:

Examensarbete E.Gränse & L.Pontell.doc (D10273613)
130521 Effects of 12 weeks exposure to probiotic.pdf (D8166507)
Examensarbete C.Sörensen.pdf (D8124135)
gb_vi.docx (D17753738)

Instances where selected sources appear:

7

APPENDIX III

CERTIFICATE - II

This is to certify that this dissertation work titled "E**ffect of custom-made probiotic chocolates on *Streptococcus mutans*, plaque pH, salivary pH and buffering capacity in children- A radomized controlled trial"** of the candidate **Dr.Vijayasankari V** with registration number ..... for the award of "**Master of Dental Surgery"** in the branch **of Pedodontics and Preventive Dentistry**. I personally verified the urkund.com website for the purpose of plagiarism Check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **7%** percentage of plagiarism in the dissertation.

Guide & Supervisor sign with Seal.

APPENDIX – IV

K.S.R Institute of Dental Science and Research

Tiruchengode -637215

CONSENT FORM

I ....., as legally responsible parent/guardian give my consent for the participation of my child ....., standard..... In the study title "Effect of custom made probiotic chocolates on *Streptococcus mutans*, plaque pH, salivary pH and buffering in children- A randomised controlled trial'. Dr. Janani RG discussed with me to my satisfaction, the procedures, possible discomforts, as well as possible benefits of the study. I have read this consent and have clearly understood the procedures to be performed on my child.

Legally responsible parent/guardian: ..... Date: .....

Address: .....

Contact number:

I certify that I explained the above information to the parent/guardian, before requesting his or her signature.

Signature of the dentist: ..... Date: .....

APPENDIX – V

K.S.R பல் மருத்துவக் கல்லூரி மற்றும் ஆராய்ச்சி மையம்

திருச்செங்கோடு -637215

தகவலறிந்த ஒப்புதல் படிவம்

பெயர் _____ வயது _____ ஆண்/பெண் ஆகியநான்என்மகன்/மகள் _____ ,வயது ___ அவர்களை மருத்துவர் அவர்களின் ஆராய்ச்சிக்கு உட்படுத்த அனுமதி கோரப்பட்டுள்ளது. இவ்வாராய்ச்சிப்பற்றி விளக்கங்களும், முறைகளும் நான் படித்துப்பார்த்தேன்/படித்துக்காட்டப்பட்டது. எனது சந்தேகங்களுக்கு தெளிவாகவிளக்கம்அளிக்கப்பட்டதுஎனவேநான்எனதுமகனை./மகளை இவ்வாராய்ச்சியில் பங்கெடுக்க அனுமதி அளிக்கிறேன்.

இடம்:

தேதி : (பெற்றோர் கையொப்பம்)

APPENDIX – VI

LIST OF SCHOOL NAMES

- **School 1-** MDV Higher Secondary School
- **School 2-** Kootapalli Government School

APPENDIX – VII

ABBREVIATION

pH	Potential of Hydrogen
S.mutans	*Streptococcus mutans*
mins	minutes
L.	*Lactobacillus*
Hcl	Hydrochloric acid
mL	Millilitre
FDA	Food and Drug Administration
CFU	Colony forming units
°C	Celsius
µm	Micrometer
µg	Microgram
g	Gram
mg	Milligram

APPENDIX –VIII

DECAYED-MISSING-FILLED INDEX (DMF) - Klein, Palmer and Knutson in 1938

- DMF teeth index (DMFT) which measures the prevalence of dental caries/Teeth.

D component:

Used to describe (Decayed teeth) which include:

1. Carious tooth.
2. Filled tooth with recurrent decay.
3. Only the root is left.
4. Defect filling with caries.
5. Temporary filling.
6. Filled tooth surface with other surface decayed.

M component:

Used to describe (Missing teeth due to caries) other cases should be excluded

1. Tooth that extracted for reasons other than caries should be excluded, which include:

   a-Orthodontic treatment.

   b-Impaction.

   c-Periodontal disease.

2. Unerupted teeth.
3. Congenitally missing.
4. Avulsion teeth due to trauma or accident.

F component:

Used to describe (Filled teeth due to caries). Teeth were considered filled without decay when one or more permanent restorations were present and there was no secondary (recurrent) caries or other area of the tooth with primary caries. A tooth with a crown placed because of previous decay was recorded in this category. Teeth stored for reason other than dental caries should be excluded, which include:

1. Trauma (fracture).
2. Hypoplasia (cosmetic purposes).
3. Bridge abutment (retention).
4. Seal a root canal due to trauma.
5. Fissure sealant.
6. Preventive filling.

WHO modification of DMF index, 1987

- All third molars included
- Initial white spot lesion – counted as decay
- Above 30 years of age, tooth lost due to any reason – counted as missing

***def in*dex - Gruebbel, Morris and Knutson - 1943**.

The initials of the index were definedas follows:

- *d* = decayed primary teeth indicated for filling,
- *e* = decayed primary teeth indicated for extraction,
- *f* = filled primary teeth.

The missing teeth are ignored, because in children it is difficult to make sure whether the missing tooth was exfoliated or extracted due to caries or due to serial extraction. In 1950 Jackson suggested the dmf index; the initials of which had exactly the same meaning for primary as the letters of the DMF index had for permanent teeth. Jackson recommended that the index be used for a full mouth dentition from 3 to 5 years inclusive for primary molars from 3 to 8 years inclusive.